APPROACHES FOR THE DETERMINATION OF PHENOLIC COMPOUNDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND CAPILLARY ELECTROCHROMATOGRAPHY

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NOMENCLATURE

ACN	acetonitrile
CE	capillary electrophoresis
CEC	capillary elecrochromatography
CRA	carbazole-9-*N*-acetic acid
DAD	diode array detector
3,4-DBA	3,4-dimethoxybenzoic acid
DMAP	4-dimethylaminopyridine
DMF	*N,N*-dimethylformamide
EDAC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EOF	electroosmotic flow
4-HBA	*p*-hydroxybenzoic acid
HPLC	high-performance liquid chromatography
IEC	ion-exchange chromatography
IPC	ion-pair chromatography
k'	chromatographic retention factor
k'*	retention factor for charged analyte in CEC
K	thermodynamic equilibrium constant
LC	liquid chromatography
LIF	laser-induced fluorescence
LOD	limit of detection

MeOH	methanol
N	plate number
NPC	normal phase chromatography
ODS	octadecyl-silica
RPC	reversed- phase chromatography
SEC	size-exclusion chromatography
SOS	sodium octyl sulfate
SPE	solid phase extraction
TFA	trifluoroacetic acid
t_0	migration time of a neutral marker
t_R	retention time of a solute
UV	ultra-violet
w_h	peak width at half height
ΔG°	free energy change
ϕ	phase ratio
u_{eo}	electroosmotic flow velocity
u_{ep}	electrophoretic velocity of the solute
u_s	solute migration velocity

CHAPTER 1

SOME BASIC PRINCIPLES OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND CAPILLARY ELECTRO-CHROMATOGRAPHY METHODOLOGIES. RATIONALE AND SCOPE OF THE STUDY

Introduction

Phenolic compounds are widely distributed in nature. Among them, two opposite categories exist, one is nutritional and the other belongs to pollutants. Our research efforts in this dissertation were focused on the introduction of analytical separation methods for these two phenolic categories. The methods are based on high-performance liquid chromatography (HPLC) and capillary elecrochromatography (CEC). For clarity of the overall dissertation, specific background on the nature of the phenolic compounds under investigation can be found in Chapters 2 and 3. To be more specific, Chapter 2 deals with the HPLC of phenolic choline esters which are an integral part of the human diet while Chapter 3 is concerned with the CEC of substituted phenols that are considered as the metabolites of various pesticides.

The rationale of using two different separation techniques, i.e., HPLC and CEC, resides in the wide differences among the phenolic structures. That is, the polarity of

the solutes of interest was quite broad, thus necessitating different separation methods. Therefore, it is the aim of this chapter to provide an overview of some basic principles of HPLC and CEC methodologies.

An Overview of The HPLC Methods Used in This Study

Thus far, HPLC is the most widely used of all of the analytical separation techniques, with annual sales of HPLC equipment approaching the billion-dollar mark (1). The reason for the popularity of the method is its sensitivity in determining small amounts of substance, its applicability to a wide variety of species, the precision of the instrumentation and the flexibility in choosing different mobile phases, stationary phases and elution methods for separation. However, new separation technologies, e.g., capillary electrophoresis (CE) and CEC have emerged as complementary techniques to HPLC to solve some separation problems that are not easy to address by HPLC.

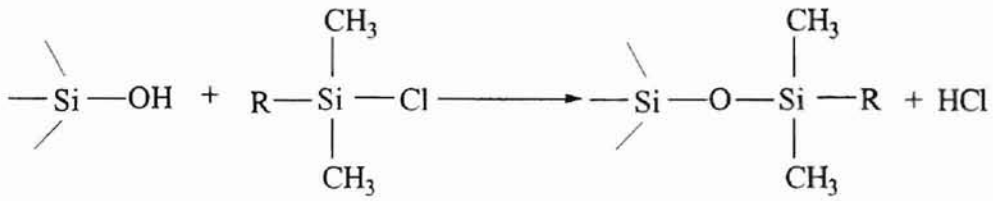
The flow of mobile phase in an HPLC column is driven by a high-pressure pump. The maximum pressure is usually limited to 6000 psi (40 MPa) which limits the design of column and ultimately separation (2). With pumped flow (also called laminar flow), the flow velocity profile is parabolic which reduces the efficiency of the column, since the velocity in the center of any channel can be twice as fast as that along the walls of the column.

Different elution methods can be developed according to the different retention factor (k') values of solutes. In isocratic elution, the composition of the mobile phase remains the same during the chromatographic runs. For compounds with a wide range of

k', the elution time could be very long and band broadening will occur. In gradient elution, mobile phase composition is varied during analysis from low to high eluting strength. Thus, compounds with a wide range of k' can be separated in a reasonable time. This flexibility makes gradient elution used most widely in practice.

According to the polarity of the stationary phase, HPLC can be divided into reversed- phase chromatography (RPC) and normal phase chromatography (NPC). 80% ~ 90% of separations are carried out by RPC in which the mobile phase is more polar than the stationary phase. The most widely used stationary phases are nonpolar microparticles based on silica support bonded with non-polar functions such as alkyl (-CH_3, -C_4H_9, -C_8H_{17} and -$C_{18}H_{37}$) or phenyl (C_6H_5) groups, see Fig. 1. For steric reasons, it is not possible for all the silanol groups on the silica surface to react with the function groups, and usually only about 45% of the silanols will be bonded (3). Unreacted acidic silanol groups may cause tailing of basic solutes. A small silating agent, such as trimethylchlorosilane [$(CH_3)_3SiCl$] is often added to react with the unreacted silanol, a process known as end-capping (4), see Fig. 2.

In addition, polymer-based stationary phases (e.g., rigid macroporous polystyrene divinylbenzene) are also used in RPC (5,6). They have some remarkable advantages over silica-based stationary phases. With non-polar functional groups covalently bonded to the polymer backbone, undesirable residual surface functional groups such as silanol sites or some metal impurities are diminished, and this leads to true reversed-phase separation. The peak tailing normally associated with the separation of basic compounds by silica-based stationary phase are prevented significantly (7). However, it should be noted that silica-based PRC columns are still used far more extensively than polymer-based RPC columns because of their much higher separation efficiency.



Silica | Alkyl or phenyl dimethylchlorosilane | Bonded Silica

R: alkyl or phenyl groups

Figure 1. Reaction of silica gel with a monochlorosilane to produce a reversed - phase stationary phase.

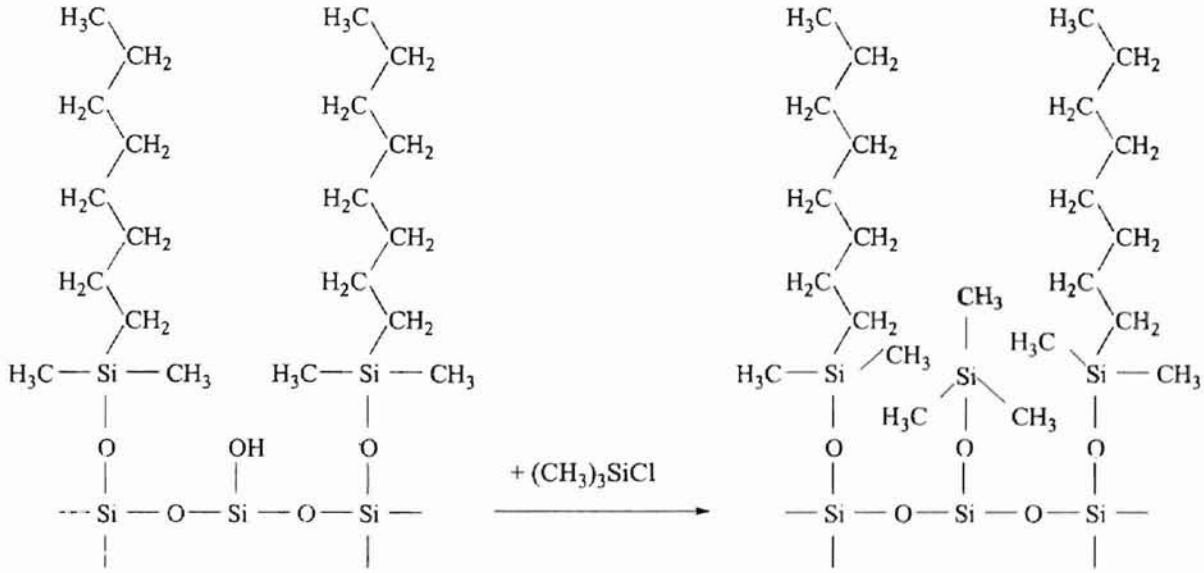


Figure 2. End capping process: Unreacted silanols are removed by reacting with trimethylchlorosilane.

Depending on the polarity of solutes, three categories of mobile phases are used in RPC: (i) Plain aqueous eluents such as pure water or buffered aqueous mobile phases are the weakest eluents used for polar solutes, (ii) mixed organic-aqueous eluents such as

methanol-water or acetonitrile-water are medium eluent strength used for slightly polar solutes and (iii) non-aqueous mobile phases like pure methanol or acetonitrile are the strongest eluents used for non-polar substances. Retention in RPC increases with increasing non-polar character of the solutes and decreasing concentration of organic solvent in the mobile phase.

Ion-pair chromatography (IPC) expands the utility of RPC to ionic solutes. The stationary phase in IPC is the same as that in RPC, and the mobile phase is a buffered hydro-organic eluent containing an ion-pairing agent. For instance, the ion-pairing agent will be a cation (I^+) for the retention and separation of an anionic solute (S^-). The mechanisms of solute retention in IPC are still controversial. Generally, there are two possible processes (8, 9): (i) adsorption of ion pairs to the hydrophobic sorbent, or (ii) formation of an in-situ ion-exchanger column (dynamic ion-exchange). In the first process, an ion-pair is formed in the mobile phase between the ion-pairing agent and the solute and is then retained in the stationary phase according to the following equilibria:

$$I_m^+ + S_m^- \longleftrightarrow (I^+S^-)_m$$

and

$$(I^+S^-)_m \longleftrightarrow (I^+S^-)_s$$

where the subscripts "m" and "s" denote mobile phase and stationary phase respectively. In the second process, an ion-pair is formed between the ion-pairing agent and mobile phase counter-ions (M^-) and is retained in the stationary phase followed by an ion-exchange between the solute and the mobile phase counter-ions according to the following equlibria:

$$(I^+M^-)_m \longleftrightarrow (I^+M^-)_s$$

and

$$S^- + (I^+M^-)_m \longleftrightarrow M^- + (I^+S^-)_s .$$

Although the two processes are different, they lead to similar predictions for solute retention: Increasing the concentration of the ion-pairing agent in the mobile phase will increase the retention of the ionic solutes of opposite charge.

In normal phase chromatography, the mobile phase is less polar than the stationary phase which is normally based on bare silica or alumina or silica bonded with polar functions such as amino [-$(CH_2)_nNH_2$, where n is 3 or 4], cyano [-$(CH_2)_3C{\equiv}N$], and diol groups [-$(CH_2)_3OCH_2CH(OH)CH_2OH$], see Fig.3 (4). Silica has the highest polarity. For basic compounds such as amines, which are strongly retained on the silica, it is better to use alumina. Also, the chemically bonded stationary phases are significantly less polar than silica and alumina and therefore will result in less retention than that can be seen on silica or alumina columns. Besides, retention in NPC increases with increasing polar character of the solutes and increasing concentration of organic solvent in the mobile phase. Water is the strongest eluent while pure organic solvent is the weakest one.

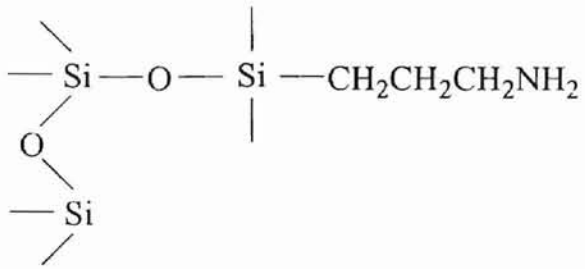


Fig.3 Structure of aminopropyl silica

Three different HPLC methods, RPC, NPC and IPC were used in our investigation. They proved useful for the separation of the phenolic choline esters and their fragments, i.e. phenolic acid, choline and betaine.

Other commonly used HPLC methods include ion-exchange chromatography (IEC) and size-exclusion chromatography (SEC) (10). IEC is best to separate and analyze ionic compounds with the help of ion-exchangers as the stationary phases whose materials carry positive or negative electric charges. The charges are balanced by an elution stream containing counter-ions of opposite sign which is similar to the sample sign. Thus, the sample can be exchanged with the counter-ion. In SEC, the stationary phase is a porous material. Molecules are separated on the basis of their sizes. Large molecules are excluded from all the pores and they elute first. Small molecules can penetrate most of the pores and they elute last. Molecules of medium size can explore part of the pores and are eluted between very large and very small molecules.

A dimensionless measure of retention in interactive chromatography (e.g. RPC, NPC, IPC and IEC) is the retention factor k' which is given by:

$$k' = \frac{\text{Number of moles of solute in stationary phase}}{\text{Number of moles of that solute in mobile phase}} = \frac{V_s C_s}{V_m C_m} = \phi K \qquad (1)$$

where V_s, V_m, C_s and C_m are volume of stationary phase, volume of mobile phase, concentration of stationary phase and concentration of mobile phase, respectively, and ϕ is the phase ratio and K is the thermodynamic equilibrium constant for the distribution of solute between mobile and stationary phase (11). K is related to the free energy change ΔG° for the solute transfer between the two phases by the equation

$$\Delta G^\circ = -RT\ln K = -2.303RT\log K$$

where R and T have their ususal meanings. So, k' is related to ΔG° by the equation

$$\log k' = \text{Log}\phi - \frac{\Delta G^{\circ}}{2.303RT} \qquad (2)$$

A practical expression for measuring k' from the chromatogram is

$$k' = \frac{t_R - t_0}{t_0} \qquad (3)$$

where t_R and t_0 are the retention time of the solute and that of an unretained solute (neutral marker). The estimation of separation efficiency (i.e., plate number N) can also be calculated from the chromatogram by

$$N = 5.54 \left(\frac{t_R}{w_h} \right)^2 \qquad (4)$$

where w_h is the peak width at half height (12).

The most popular detection system for HPLC is UV-Visible absorbance detector because many compounds in solution absorb visible or UV light. Other detection methods include fluorescence, refractive index, electrochemical, mass spectroscopy and radioactivity (11).

CEC Instrumentation and Separation Principles

Capillary electrochromatography is a relatively new micro-column technology for separation (13). It combines the unique selectivity of the stationary phase of HPLC to the

high separation efficiency and differential electomigration of CE. CEC employs open or packed fused-silica capillaries (10 - 100 μm I.D.) with retaining frits. It uses high voltage (10 - 30 KV) and the capillary efficiently dissipates the heat. CEC is also characterized by small sample requirement that makes it affordable for the use of expensive solvents, reagents and samples of specific properties that need to be handled in tiny quantity.

The instrumentation of CE/CEC is relatively simple compared to that of HPLC. It is composed of a high voltage power supply, two buffer reservoirs with a platinum electrode in each reservoir, fused silica capillary, a detector similar to that used in HPLC with some slight modifications in that it is on column detection and a data acquisition system. Figure 4 shows a typical CE/CEC instrumentation.

The driving force for the mobile phase and solute transport in CEC is the electroosmotic flow (EOF), which is the flow of the bulk electrolyte solution under the influence of an electric field. In CEC, the inner surface of the capillary wall and the stationary phase particle surface are positively or negatively charged under a certain electrolyte condition. Electrolyte counter-ions will accumulate near the charged surface by electrostatic forces and a compact region will form. Some of the ions in the compact region will migrate further into the solution due to thermal motion and a diffusion region will occur. Under the influence of an electric field, ions in the diffusion region will migrate toward the oppositely charged electrode. The electrolyte solution is dragged along by these solvated counter-ions, thus causing the EOF. Figure 5 shows that the overall EOF in CEC results from the pumping action of both the capillary wall and each particle.

Figure 6a shows a flow velocity profile in a packed bed with EOF. In

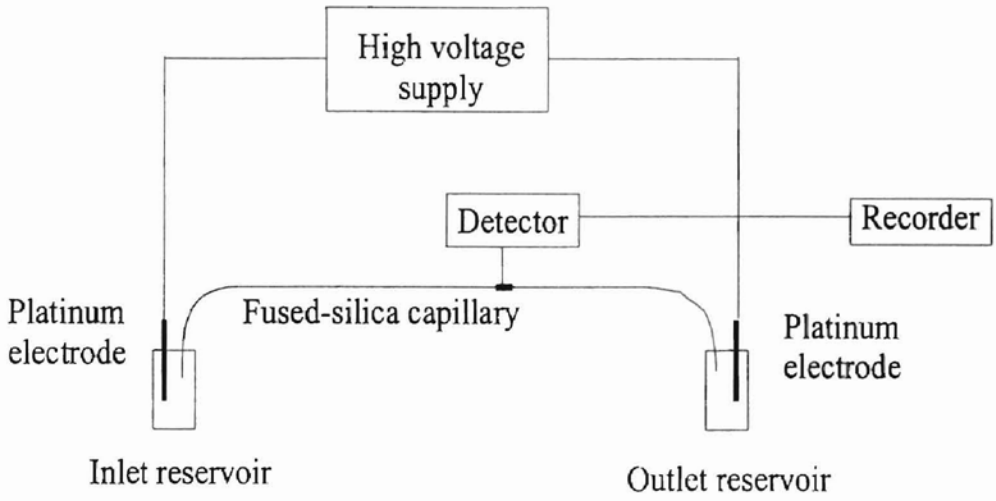


Figure 4. A typical CE/CEC instrumentation.

channels between 0.1μm and 150 μm, EOF velocity is independent of the channel width which means packing imperfection or unevenness of the stationary phase particle size do not change the flow profile. The square profile maintains the samples in a narrow band. Figure 6b shows a flow velocity profile in a packed bed with pumped flow. The flow in the center is larger than that along the wall and this will cause band broadening. Thus, the flat flow profile of EOF allows CEC to generate higher separation efficiency than HPLC. Typical efficiency with 5 μm particles is 100,000 - 150,000 plates/m in CEC as opposed to 60,000 - 80,000 plates/m in HPLC.

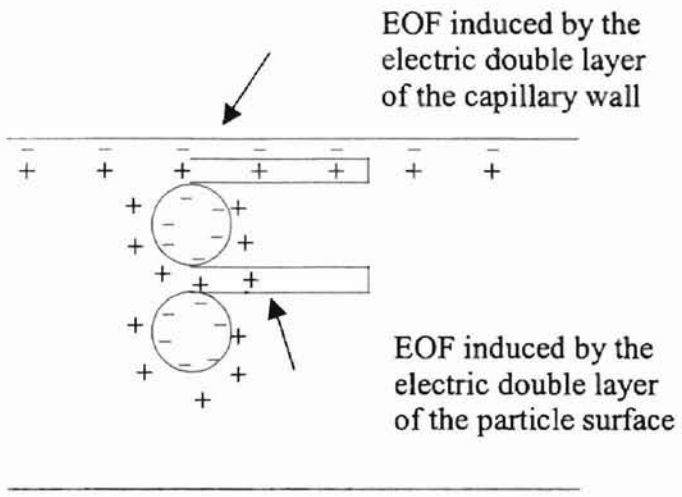


Figure 5. Electroosmotic flow in CEC.

Figure 6. (a) A flow velocity profile in a packed bed with EOF ; (b) a flow velocity profile in a packed bed with pumped flow.

In CEC, neutral solutes are separated on the basis of differences in partitioning between the mobile and stationary phases as in chromatography while charged solutes are separated by both electrophoretic migration and chromatographic partitioning. For neutral solutes, retention factor k' is defined in the same way as in chromatography and can be calculated from the electrochromatogram by the equation given above (see eqn 3). On the other hand, retention factor of charged solutes is given by a different equation

$$k'^{*} = \left[\frac{(u_{ep} + u_{eo})^2}{(u_{eo} \cdot u_s)}\right] - 1 \qquad (5)$$

where u_{ep} is the electrophoretic velocity of the solute, u_{eo} is the electoosmotic velocity and u_s is the solute migration velocity.

The equation describing separation efficiency N for CEC is the same as that in chromatography, see eqn 4.

Up to now, packed columns with silica-based stationary phases are the most widely used in CEC. The preparation on these capillary columns involves 2 key steps: (i) the fabrication of retaining frits and (ii) the packing of the stationary phases into the capillary columns. Both steps require considerable experimental skills (14). The frits are usually made by siphoning a tiny amount of silica into the column followed by sintering the silica in a Bunsen burner or a CE window maker. The heating needs to be controlled. Not enough heating may cause the frit to be too fragile to hold the packed particles inside the column, while overheating may cause the silica to consolidate too tight, and that will hinder liquid flow. Also, heating changes the characteristics of the packing material at the frit position, creating non-homogeneity at the frit. This can contribute to the nonuniformity in EOF, which can lead to bubble formation. The delivering of packing material into the capillary column is made by different methods: slurry pressure packing, packing with supercritical CO_2, electrokinetic packing, using centripetal forces, and packing by gravity (15). The efficiency of column packed by these different methods varies considerably. Slurry pressure packing is the most widely used, but some reported that other methods could offer high efficiency (15).

Another thing worthy to mention is that the octadecyl-silica (ODS) used in CEC is different from the ODS used in HPLC. OH groups in the latter are converted to the greatest extent with alkyl groups. While in CEC, parts of the OH groups are reacted and the rest are left unreacted intentionally in order to produce the EOF necessary to transport the flow of the mobile phase and solute through the column. So, in practice, many laboratories make the ODS for CEC by themselves to meet their specific needs.

Because of the difficulty associated with the preparation of a packed column, continuous beds or monolithic columns for CEC are receiving more and more attention (14). They are characterized with not only the simplicity of their in-situ preparation but also by the large amount of choices for the monolithic media. Monoliths prepared from aqueous monomer solution and dispersions have had some success (16, 17, 18), but the greatest limitation is the poor solubility of some polymerizable monomers in water. Organic solvents possess a wide range of polarity and can dissolve most monomers. Thus, porous polymer monoliths prepared using organic solvents and mixtures have presently become the focus of intensive research. Acrylamide-based, methacrylate ester-based and polystyrene-based monoliths are being studied extensively now. The preparation of a monolithic capillary for CEC is relatively simple (2): The capillary is first filled with a prepolymer cocktail, including a porogen (a solvent used to control the porosity of the final polymer), monomers to produce hydrophobic retention, cross-linking agents and charge carriers. Polymerization is then initiated in a thermostatic bath for some time and followed by washing using a syringe pump.

On column UV-Vis absorbance detector is the most commonly used detection method in CE/CEC because of its simplicity and low price (20). However, due to the small optical pathway of the detection cell of the capillary itself, UV detection lacks

sensitivity. Electrochemical detection is the most sensitive. Detection at nanomolar level of electroactive substance has been reported (21). Due to the complexity of CE/CEC instrumentation, electrochemical detection has not yet been commercialized. Fluorescence detection, especially laser induced fluorescence (LIF) detection has been considered to be the best way to overcome the low detection sensitivity associated with UV-Vis detection in CE/CEC. It uses laser as the excitation source. A laser emits a highly coherent and powerful light which can be focused on the capillary precisely and almost 100% of the energy can be utilized.

Rationale and Scope of the Study

Phenolic compounds are important synthetic and natural products implicated in various biological, industrial and environmental processes. These diverse functions of phenolic compounds are paralleled by their varying physico-chemical properties such as polarity, volatility, detectability, etc. On this basis, it becomes obvious that there is no one single separation method or technique that can accommodate the separations and detection of all phenolic species. This is further complicated by the lack of authentic standards for some phenolics, a condition that make their accurate determination rather difficult in most standard laboratories where sophisticated spectroscopic techniques (e.g., mass spectrometry and NMR) may not be available. Thus, the need for ways to modify the phenolic species to fragments reflective of the individual or total phenolics in the sample and base their determination on these fragments.

As stated at the beginning of this chapter, our studies dealt with two different groups of phenolics: Chapter 2 involved the HPLC of phenolic choline esters which are

the bound forms of phenolic acids found in some plants, and Chapter 3 focused on the CEC of substituted phenols which are the transformation products of important pesticides. While HPLC proved very adequate for the determination of phenolic choline esters, which are found in some plants in rather large quantities, the substituted phenols which are usually found in environmental matrices (e.g., soil, water) at low concentration levels necessitated the use of CEC which usually incorporates laser induced fluorescence (LIF) detection which is the most sensitive optical detection method now available.

Due to the lack of authentic standards for phenolic choline esters, we have developed chemical and enzymatic degradation processes to produce fragments that are reflective of the parent phenolic choline esters and for which authentic standards are available from commercial sources. In fact, phenolic choline esters can be degraded in alkaline solutions to phenolic acids (available as standards), which can be used in the qualitative and quantitative analysis of the parent phenolic choline esters. Also, the base hydrolysis of phenolic choline esters produces choline, which can be further modified to betaine by the action of the enzyme choline oxidase, thus permitting the quantitative determination of total phenolic choline esters via betaine, which is available as a pure standard.

For the substituted phenols, which are the subject of Chapter 3, we have implemented a novel precolumn derivatization scheme to convert the analytes into fluorescent derivatives and facilitate their subsequent sensitive detection by LIF. The precolumn derivatization not only enhanced the detectability of the various substituted phenols but facilitated their separation. This is particularly true for the isomers of monochlorophenol.

The significance of the studies resides in advancing the methodologies of both HPLC and CEC, which are considered complementary to each other. The study provided separation and detection systems that will find general use in the analysis of phenols and related compounds as well as in other areas of the life science.

References

1. D.A. Skoog, F.J. Holler and T.A. Nieman, *Principles of Instrumental Analysis, fifth edition*, Saunders College Publishing, Philadelphia, 1998, pp. 726-766.
2. I.S. Krull, R.L. Stevenson, K. Mistry and M.E. Swartz, *Capillary Electrochromatography and Pressurized Flow Capillary Electrochromatography An Introduction,* HNB Publishing, New York, 2000, pp. 1-90.
3. B.L. Karger and R.W. Giese, *Anal. Chem.* 1978, *50*, 12.
4. A.Weston and P.R.Brown, *HPLC and CE Principles and Practice,* Academic Press, San Diego, 1997, pp. 1-50.
5. U.D. Neue, *HPLC Columns Theory, Technology, and Practice,* Wiley-Vch Publishers, New York, 1997, pp.106-127.
6. G. Szepesi, *How to Use Reverse – Phase HPLC,* VCH Publishers, New York, 1992, pp. 1-61.
7. Interaction Catalog, Interaction Chemicals Inc., Mountain View, 1989, pp.3.
8. W.R. Melander and Horváth, *Ion-Pair Chromatography,* M.T.W. Hearn Ed., Marcel Dekker, New York, 1985, Chapter 2.
9. L.R. Snyder, *Chromatography, Journal of Chromatography Library,* 1992, *51 A,* 1.
10. R.W. Yost, L.S. Ettre and R.D. Conlon, *Practical Liquid Chromatography An Introduction,* Perkin-Elmer Corp., Norwalk, 1980, pp. 93-118.
11. W.J. Lough and I.W. Wainer, *High Performance Liquid Chromatography Fundamental Principles and Practice,* Blackie Academic & Professional Publishing, London, 1995, pp. 1-142.

12. C.F. Poole and S.K. Poole, *Chromatography today,* Elsevier Science Publishers, 1991, pp. 1-95.

13. P. Camilleri, *Capillary electrophoresis Theory and Practice,* CRC press LLC, Florida, 1998. pp. 1-18.

14. F. Svec, E.C. Peters, D. Sýkora and J.M.J. Fréchet, *J. Chromatogr. A,* 2000, *887,* 3-29.

15. L.A. Colón, T.D. Maloney and A.M. Fermier, *J. Chromatogr. A,* 2000, *887,* 43-53.

16. P.A. Novotny, M.V., *Anal. Chem.,* 1997, *69*, 4499-4507.

17. S. Hjertén, *Biochem.* Biophys., 1962, *Suppl. 1,* 147-151.

18. M. Zhang and Z. El Rassi, *Electrophoresis,* 2001, *22,* 2593-2599.

19. G. Nouadje, J. Amsellem, B. Couderc, P. Verdeguer and F. Couderc, in *Progress in HPLC-HPCE, Volume 5 Capillary electrophoresis in biotechnology and environmental analysis,* H. Parvez, P. Caudy, S. Parvez and P. Roland-Gosselin (Eds.) Utrecht, Netherlands, 1997, pp. 49-75.

20. I.C. Chen and C.W. Whang, *J. Chromatogr.* 1993, *644,* 208-212.

21. R. Kuhn and S. Hoffstetter-Kuhn, *Capillary Electrophoresis Principle and Practice,* Springer-Verlag, Berlin. 1993.

CHAPTER II

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF PHENOLIC CHOLINE ESTERS FRAGMENTS DERIVED BY CHEMICAL AND ENZYMATIC FRAGMENTATION PROCESS. ANALYSIS OF SINAPINE IN RAPE SEED

Introduction

Phenolic acids, e.g., benzoic (C_6-C_1), and hydroxycinnamic acid (C_6-C_3) derivatives, are aromatic secondary plant metabolites belonging to the class of plant phenolics. Phenolic acids are found in almost every plant (1, 2), and therefore, are an integral part of the human diet. In fact, phenolic acids are important components in a wide variety of fruits (e.g., white grapes, tomatoes, apples, pears, cherries, plums, peaches, apricots, blueberries, etc.), in vegetables (spinach, cabbage, asparagus, potatoes, etc.), coffee, olive oil, wheat, corn, wines and rice to name a few crops (3, 4).

Interest in phenolic acids is related to their diversity, biological significance as secondary plant metabolites and ecological role, use as chemotaxonomic markers, impact on fruit and vegetable quality, physiological effects and various applications. Recent interest in food phenolic acids has increased greatly because of the antioxidant and free radical scavenging abilities associated with phenolic acids and their potential effects on human health (5). It is well known that diets rich in fruits and vegetables are

protective against cardiovascular diseases and certain forms of cancer (6-8), and perhaps against other diseases also.

Besides existing as free species, the majority of phenolic acids occur naturally in a wide group of combined forms. They occur in association with cyclohexane carboxylic acid (e.g., quinic acid), other organic acids (e.g., tartaric acid, malic acid), sugars (e.g., glucose), amines (e.g., choline as in sinapine and its derivatives), and also linked to other polyphenols (e.g., flavonoids) (9). Among all these bound forms of phenolic acids, we are concerned here with phenolic choline esters, known as sinapines (5, 10). Phenolic choline esters are especially abundant in seeds of some glucosinolate-containing plants or crucifer seeds (11) such as rapeseed/canola (10). Typical phenolic choline esters are shown in Fig. 1.

Phenolic choline esters are important natural products, and therefore methods for their accurate determination in plants are needed. Since standard phenolic choline esters are not available in commercial markets, the quantitative determination of phenolic choline esters in plants remains a problem. As shown in Fig. 1, phenolic choline esters can be hydrolyzed in basic solution to choline and phenolic acids, the representative of which are 4-hydroxybenzoic acid (4-HBA), 3,4-dimethoxybenzoic acid (3,4-DBA), *p*-coumaric acid, ferulic acid and sinapic acid (12). Also, choline can be enzymatically converted to betaine (13), see Fig. 1. Since the standards for the phenolic acids and betaine are readily available, the amount of choline esters can be measured by determining the amount of phenolic acids or choline after hydrolysis of the rapeseed extract. This chapter is concerned with developing HPLC methods for the determination of phenolic choline esters based on their chemical and enzymatic fragments, which are reflective of the individual and total phenolic choline esters.

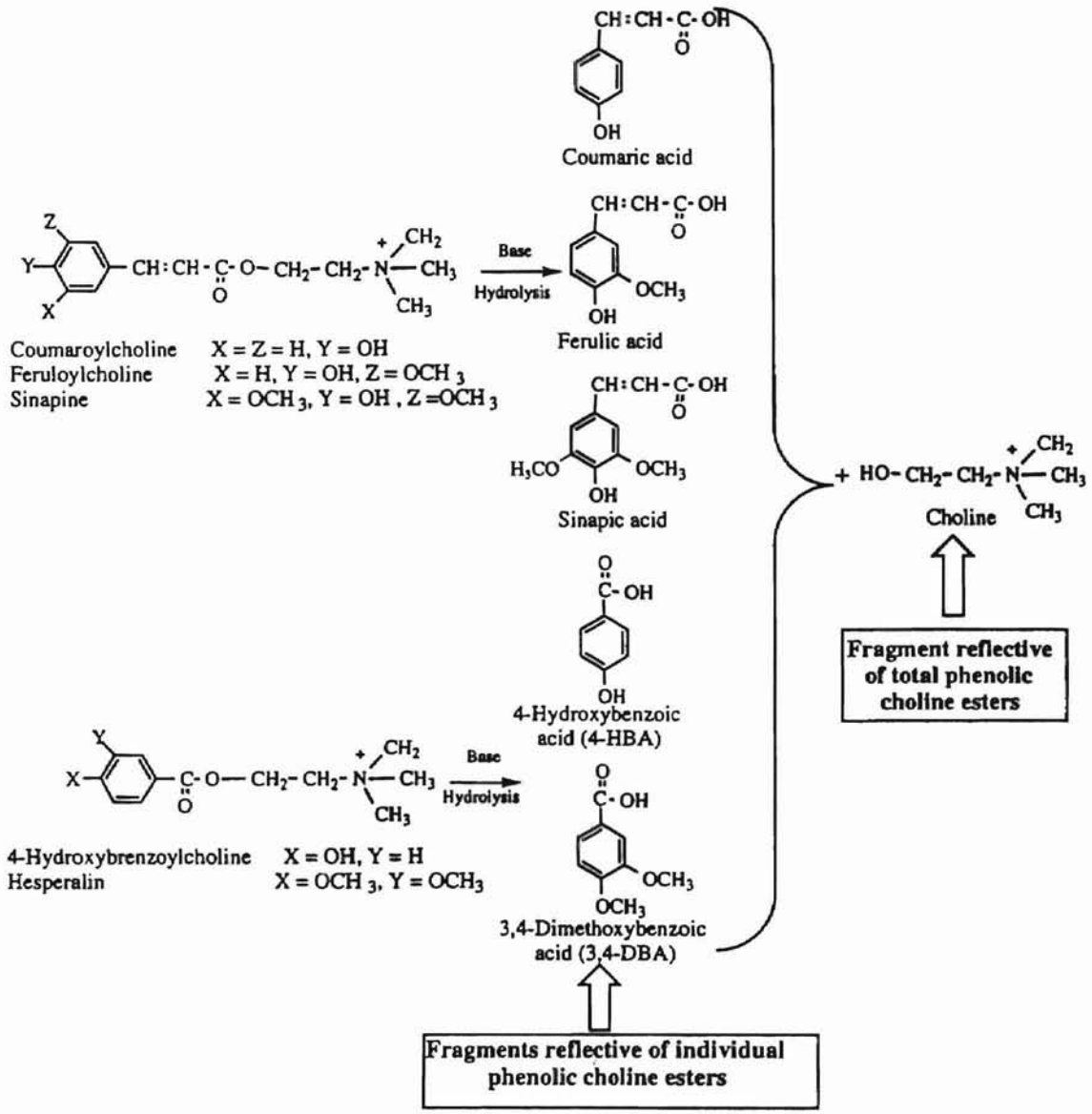

Coumaric acid
Coumaroylcholine X = Z = H, Y = OH
Feruloylcholine X = H, Y = OH, Z = OCH 3
Sinapine X = OCH 3, Y = OH , Z =OCH 3
Base
Hydrolysis
Ferulic acid
Sinapic acid
Choline
Fragment reflective of total phenolic choline esters
4-Hydroxybenzoic acid (4-HBA)
4-Hydroxybrenzoylcholine X = OH, Y = H
Hesperalin X = OCH 3, Y = OCH3
3,4-Dimethoxybenzoic acid (3,4-DBA)
Fragments reflective of individual phenolic choline esters
Choline oxidase
Betaine aldehyde
(Fast)
Betaine
(Slow)

Figure 1. Structures of some sinapines found in crucifer seeds. Also shown in this figure are the corresponding phenolic acids, choline and betaine fragments obtained by base hydrolysis and enzymatic processes. While the phenolic acids are reflective of the individual sinapines and can be used for the quantitative determination of each phenolic choline esters, choline and its enzymatic derivative betaine can be used for the quantitative determination of total phenolic choline esters in plant extracts.

Experimental

Reagents and Materials

Standards of choline, betaine, 4-hydroxybenzoic acid (4-HBA) and 3,4-dimethoxybenzoic acid (3,4-DBA) as well as choline oxidase (EC 1.1.3.17) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Standards of *p*-coumaric acid, ferulic acid and sinapic acid were from Aldrich Chemical Co. (Milwaukee, WI, USA). For the structures of phenolic acids, choline and betaine, see Fig. 1. Methanol of HPLC grade was purchased from EM Science (Gibbstown, NJ, USA). Acetonitrile of HPLC grade was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Rape seed (Dwarf Essex) was from Dr. Melouk, Department of Plant of Pathology, Oklahoma State University, Stillwater, OK. C18 Bakerbond Solid Phase Extraction (SPE) cartridges were from J.T.Baker.

Instruments and Columns

The liquid chromatograph was assembled from: (i) a Model CM 4000 multiple solvent delivery system (Milton Roy, LDC division, Riviera Beach, Florida, USA), (ii) a Model 7010 sample injector (20 µL) from Rheodyne (Cotati, CA, USA), (iii) a diode array detector (DAD) Model HP 1040A from Hewlett-Packard (Waldbronn, Germany) whose spectral range is from 190 nm to 400 nm and (iv) a personal computer equipped with the software HP Chemstation for LC systems (Rev.A.04.01, Copyright Hewlett

Packard 1990-1996). The wavelength was set at 210 nm or 192 nm for sensing the column effluent with RPC or NPC, respectively.

The columns for RPC were made of stainless steel tubing of dimensions 25 cm x 4.6 mm I.D. or 15 cm x 4.6 mm I.D. packed with narrow pore C18 of 5 μm mean particle diameter from J.T. Baker Inc. (Phillipsburg, NJ, USA). The column for NPC was also made of stainless steel tubing of dimensions 15 cm x 4.6 mm I.D. and packed in-house with narrow pore bare Zorbax silica of 5 μm mean particle diameter. In all experiments, we used an analytical guard column made from stainless steel tubing of dimensions 2 cm x 2 mm I.D. (Upchurch Scientific, Murrieta, CA, U.S.A.) and dry packed in house with Zorbax C18 of 18 μm mean particle diameter.

Chromatographic Conditions

In all cases, the flow-rate was set at 1.0 mL/min. Before running the experiments, the mobile phases were first filtered through S|P™ filter paper (grade 360 qualitative from Baxter, McGaw Park, IL, USA) and then degassed in an ultrasonic bath. For gradient runs with the C18 column, mobile phase A_1 consisted of 10 mM $NH_4H_2PO_4$, pH 6.0, while mobile phase B_1 consisted of 80% MeOH and 20% mobile phase A_1. Linear gradients for 20 min were run from 100% A_1 to 100% B_1. For gradient runs with the silica column, mobile phase A_2 consisted of 98% ACN and 2% of 10 mM NH_4Cl, pH 3.6 (v/v), while mobile phase B_2 consisted of 50% ACN and 50% of 10 mM NH_4Cl, pH 3.6 (v/v). Again, linear gradients for 20 min were run from 100% A_2 to 100% B_2.

Procedures

In the following sections we describe the various step involved in the sample preparation and the quantitative determination of the various components of interest by

HPLC. Figure 2 summarizes the various steps involved in sample preparation and analysis.

Rapeseed extraction

An amount of 21.0 g of seed of rapeseed was ground thoroughly until slurry in a mortar. The slurry was then extracted with carbon tetrachloride in a Soxhlet apparatus for 4 hours and then dried overnight at room temperature (14). Next, the defatted material thus obtained was extracted twice with 50 mL of HPLC methanol each. Finally, methanol was evaporated in a Savant vapor trap (Savant, Holbrook, NY, USA) and 0.50 g of orange-colored solid was obtained.

Solid phase extraction (SPE)

The solid phase extraction (SPE) was carried out as follows: Activate the C18 SPE cartridge with 80% methanol: 20% H_2O (v/v), and then condition the cartridge with deionized water @ 0.1% (v/v) trifluoroacetic acid (TFA). Thereafter, apply the sample (0.0402 g extract in 0.80 mL of 0.050 M Na_3PO_4, pH 8.0) and elute the cartridge with 50% methanol: 50% deionized water (v/v) @ 0.1% TFA (v/v). Collect the eluent from both the sample application step and the elution step into a vial. The collected fraction was then dried from methanol and TFA in a Savant vacuum system. The solid obtained was yellow-brown.

Base hydrolysis of rapeseed extract

A small amount (see below for exact amount) of the solid extract from rapeseed was dissolved in 1.0 mL of mobile phase A_1 which consisted 10 mM sodium phosphate, pH 6.0. To this solution, 100 µl of 2.0 M NaOH were added, and the base hydrolysis was carried out for 2 hours. The hydrolyzate was then neutralized by adding 100 µL of 2.0 M HCl.

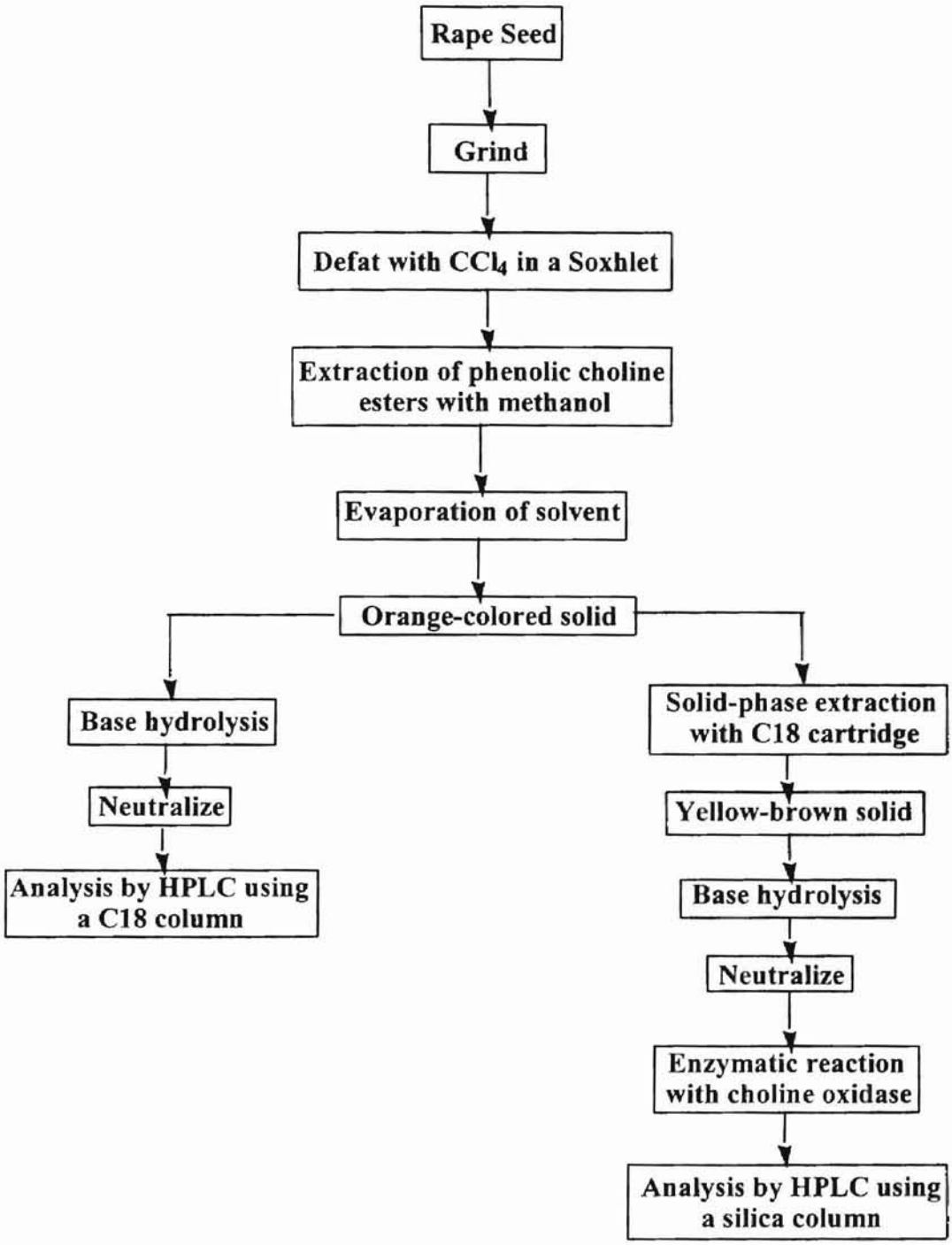


Figure 2. Steps involved in sample preparation and analysis.

Standard calibration curve

To quantitatively determine the amount of sinapic acid in the rapeseed after hydrolysis, we used ferulic acid as an internal standard and made a standard calibration curve by plotting the ratio of peak height of sinapic acid and ferulic acid versus the concentration of standard sinapic acid. The standard solution of sinapic acid was made by dissolving 0.0052 g of sinapic acid into 1.0 mL of methanol which was added to a 10 mL volumetric flask and the final volume adjusted with mobile phase A_1 until 10 mL. The final concentration of sinapic acid was 2.32×10^{-3} M. The standard solution of ferulic acid was made by dissolving 0.0098 g of ferulic acid into 1.0 mL of methanol which was added to a 50 mL volumetric flask and the final volume was adjusted with mobile phase A_1 until 50 mL. The final concentration of ferulic acid was 1.00×10^{-3} M.

The rapeseed sample was prepared as follows: 0.0037 g of rapeseed extract was added into a 25 mL volumetric flask followed by adding 2.0 mL of mobile phase A_1 and 100 μL of 2.0 M NaOH. After 2 hours, 100 μL of 2.0 M HCl was added to neutralize the solution followed by adding 300 μL of standard ferulic acid and mobile phase A_1 to reach the 25 mL mark. The solutions for the calibration curve were prepared as follows: Pipet 300 μL of standard ferulic acid into six 25 mL volumetric flasks and then add 300, 400, 500, 600, 700 and 800 μL of standard sinapic acid, respectively. Thereafter, complete the volume to 25 mL by adding mobile phase A_1. After sufficient stirring, inject the sample and standard solutions into the HPLC system.

To quantitatively determine the amount of betaine converted from choline by choline oxidase, alanine was used as an internal standard. The standard calibration curve was made by plotting the ratio of peak height of betaine and alanine versus concentration of standard betaine. The standard solution of betaine was made by weighing 0.0355 g of betaine into a 25 mL volumetric flask followed by adding 0.05 M Na_3PO_4 solution, pH 8.0, to reach the mark. The standard solution of alanine was made by weighing a small

amount of alanine into a 10 mL volumetric flask followed by adding 0.05 M Na_3PO_4 solution, pH 8.0, to reach the mark.

The rapeseed sample was prepared as follows: 0.0402 g of rapeseed extract was added into a small vial followed by adding 1.0 mL of water: methanol (3:2). After applying solid phase extraction, the yellow-brown solid was dissolved in 0.8 mL of 0.05 M Na_3PO_4 solution, pH 8, into a small vial followed by adding 100 µL of 2.0 M NaOH. After 2 hours, 100 µL of 2.0 M HCl was added to neutralize the solution. Thereafter, 200 µl of standard alanine solution and a known number of units of choline oxidase were added into the vial which was then put into a Thermolyne, Type 17600 DriBath (Dubuque, IA, USA) that remained at a constant temperature of 37°C for 18 hours.

The solutions for the calibration curve for betaine were prepared as follows: Pipet 200 µL of standard alanine solution into six small vials and then add 200, 300, 400, 500, 600 and 700 µL of standard betaine solutions, respectively. Thereafter, add 800, 700, 600, 500, 400 and 300 µL of 0.05 M Na_3PO_4, pH 8.0, respectively. Under these conditions, the total volume of each of the six solutions is 1.200 mL.

Results and Discussion

Due to the large differences in the polarity of solutes involved in this study, two different HPLC modes were utilized, i.e., RPC with C18-silica column and NPC with silica column.

Chromatographic Behavior of the Standard Phenolic Acids and Betaine

In a series of experiments aiming at determining the optimum **pH** for the separation of the phenolic acid constituents of phenolic choline esters, 20 min linear

gradient runs from 100% mobile phase A_1 to 100% mobile phase B_1. Mobile phase A_1 consisted of 10 mM $NH_4H_2PO_4$ at pH 2.5, 4.0, 4.5, 5.0 or 6.0 while mobile phase B_1 consisted of 80% MeOH and 20% solvent A_1. At pH 2.5, *p*-coumaric, ferulic and sinapic acids coeluted while at pH 4.0, 4.5 and 5.0 ferulic and sinapic acids coeluted despite the structural difference among the three acids. At pH 6.0, all the 5 acids were completely separated, see Fig. 3. In addition to structural differences, at pH 6.0, the acids are ionized to varying degree, a fact that further differentiates their partitioning between the mobile and stationary phases and brings about their separation. The optimum pH for the phenolic acids was thus set at pH 6.0. On the other hand, choline and its enzymatic derivative betaine were not retained in the pH range tested, i.e., pH 2.5-6.0

On the basis of the above experiments, incorporation of a negatively charged ion-pairing agent (e.g., sodium octyl sulfate SOS) was considered to bring about the retention of betaine and choline. Isocratic elution was conducted with a mobile phase of 10 mM ammonium phosphate, pH 6.0 at various SOS concentrations. In the absence of SOS, betaine and choline were not retained and only 4-HBA could be eluted while the other acids were very retarded. The dependence of solute retention factor k' on the concentration of SOS is shown in Fig. 4. As can be seen in this figure, the retention factor of betaine increased substantially first from 0 to 0.55 upon adding 2.5 mM SOS and then leveled off as the SOS concentration was increased. Surprisingly, choline stayed unretained in the concentration range studied (0 to 10 mM SOS). The retention factor of the five phenolic acids decreased with increasing SOS concentration. The decrease in solute retention of phenolic acids is due to the fact that the ion-pairing agent carries the same charge as the solute thus leading to solute repulsion from the stationary phase with adsorbed SOS. It is noteworthy that when surfactant concentration was at 0 mM, the retention of 4-HBA was longer than that of betaine. At 2.5 mM SOS, betaine and 4-HBA coeluted. After 5 mM, the retention of betaine became longer than that of

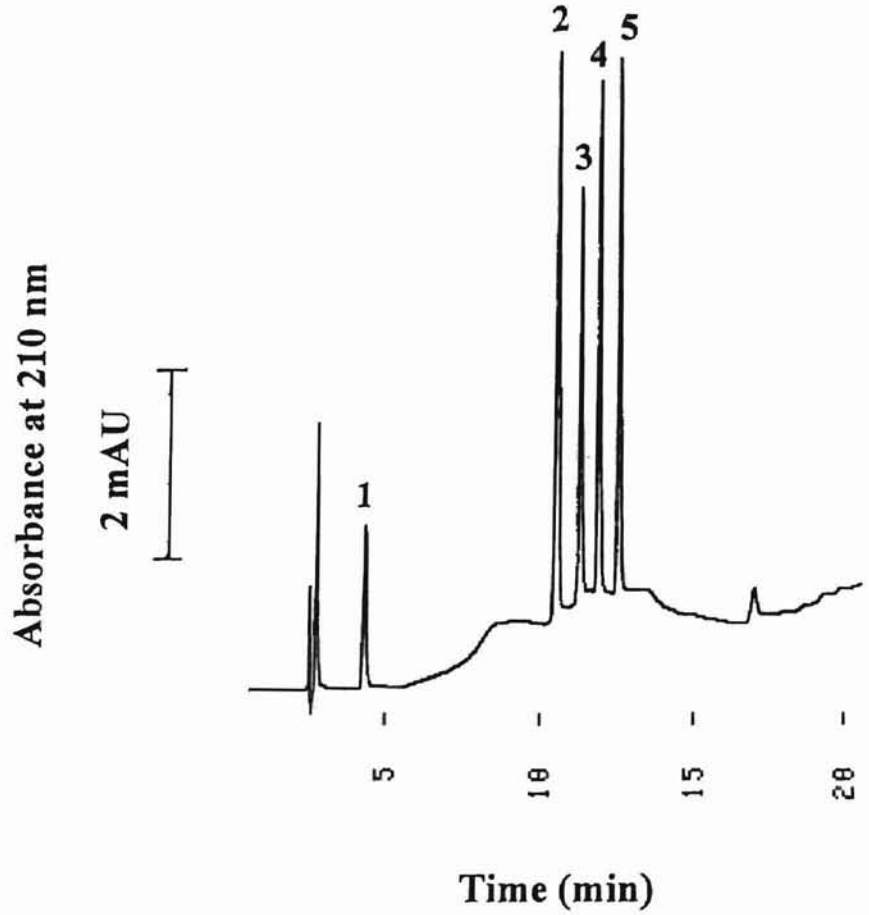


Figure 3. Chromatogram of the major phenolic acid constituents of phenolic choline esters obtained by RPC. Column, 250 x 4.6 mm I.D. packed with C18-silica; flow rate, 1.0 mL/ min; 20 min linear gradient elution from 100% A_1 to 100% B_1. Solutes: 1, 4-HBA; 2, *p*-coumaric acid; 3, 3,4-DBA; 4, ferulic acid; 5, sinapic acid.

HBA. This could be seen clearly at 10 mM, see Fig. 4. Although the ion-pairing system allowed the simultaneous analysis of the phenolic acids and betaine constituents of phenolic choline esters, the retention of betaine was not sufficient and that of 4-HBA decreased significantly making both solutes to elute near the dead time of the column where interferences of real samples may also elute.

Therefore, another chromatographic system more adequate for the analysis of choline and betaine was considered. On the other hand, the RPC chromatographic system based on gradient elution shown in Fig. 3 is very adequate for the analysis of the phenolic acid constituents. Also, the RPC system can be performed isocratically since the retention and selectivity of the system can be manipulated readily by the methanol content of the mobile phase, see Fig. 5. As can be seen in this figure, for each phenolic acid log k' is linearly related to % methanol and as expected, the absolute value of the slopes increased with the size of the molecule.

As stated above, for the separation of betaine and choline we had to use NPC with bare silica column. Figure 6 shows the chromatograms of standard choline and betaine obtained on a silica column of dimensions 15 cm x 4.6 mm I.D. run at a flow rate of 1.0 mL/min with a 20 min linear gradient from 100% solvent A_2 to 100% solvent B_2. Solvent A_2 consisted of 98% ACN and 2% of 10 mM NH_4Cl, pH 3.6 (v/v), while solvent B_2 consisted of 50% ACN and 50% of 10 mM NH_4Cl, pH 3.6 (v/v). As can be seen in Fig. 6 choline and betaine elute at ca. 9.8 and 17.7 min, respectively, and they are far removed from the column dead time where many unretained species in real samples would elute.

Determination of Sinapine in the Extract of Rapeseed via its Degradation Products: Sinapic Acid or Choline

Analysis via the base hydrolysis of the rapeseed extract

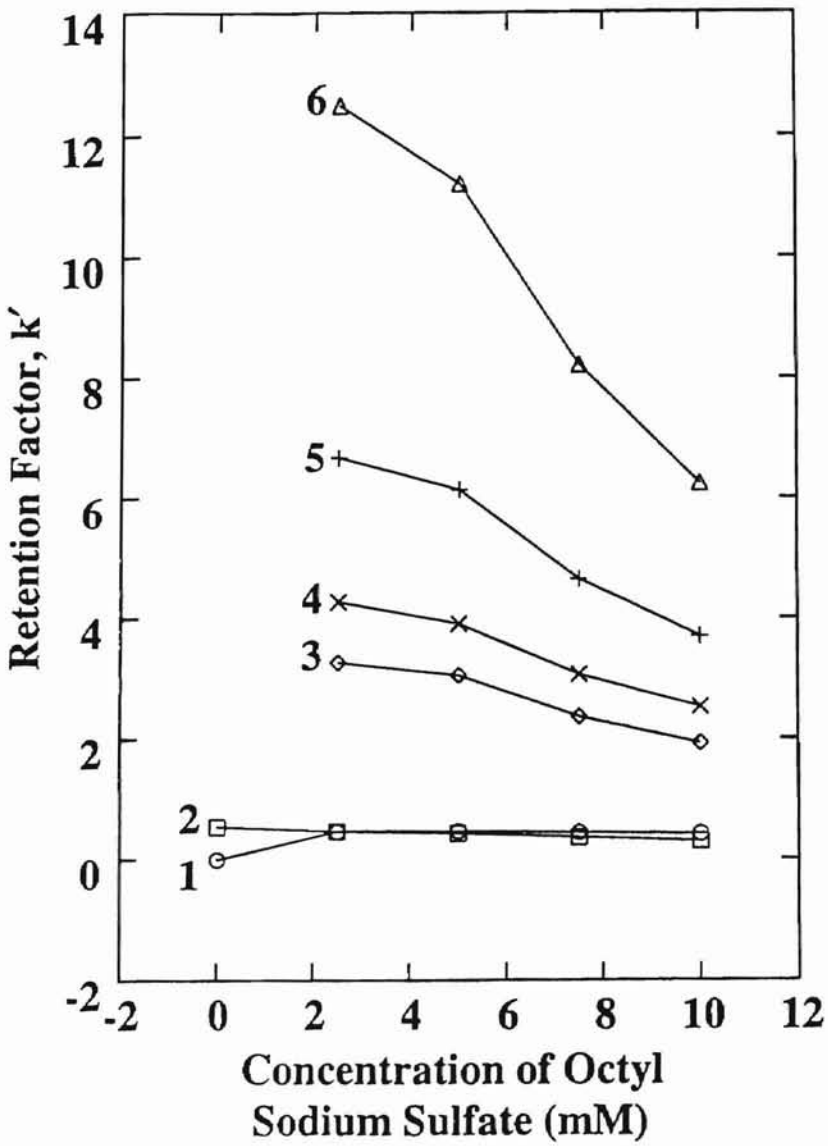


Figure 4. Plots of retention factors of betaine and some phenolic acids versus the concentration of sodium octyl sulfate (SOS) in the mobile phase. Column, 250 x 4.6 mm I.D. packed with C18-silica; flow rate, 1.0 mL/ min; isocratic elution with a mobile phase of 10 mM $NH_4H_2PO_4$, pH 6.0, at various concentration of SOS. Curves: 1, betaine; 2, 4-HBA; 3, *p*-coumaric acid; 4, 3,4-DBA; 5, ferulic acid; 6, sinapic acid.

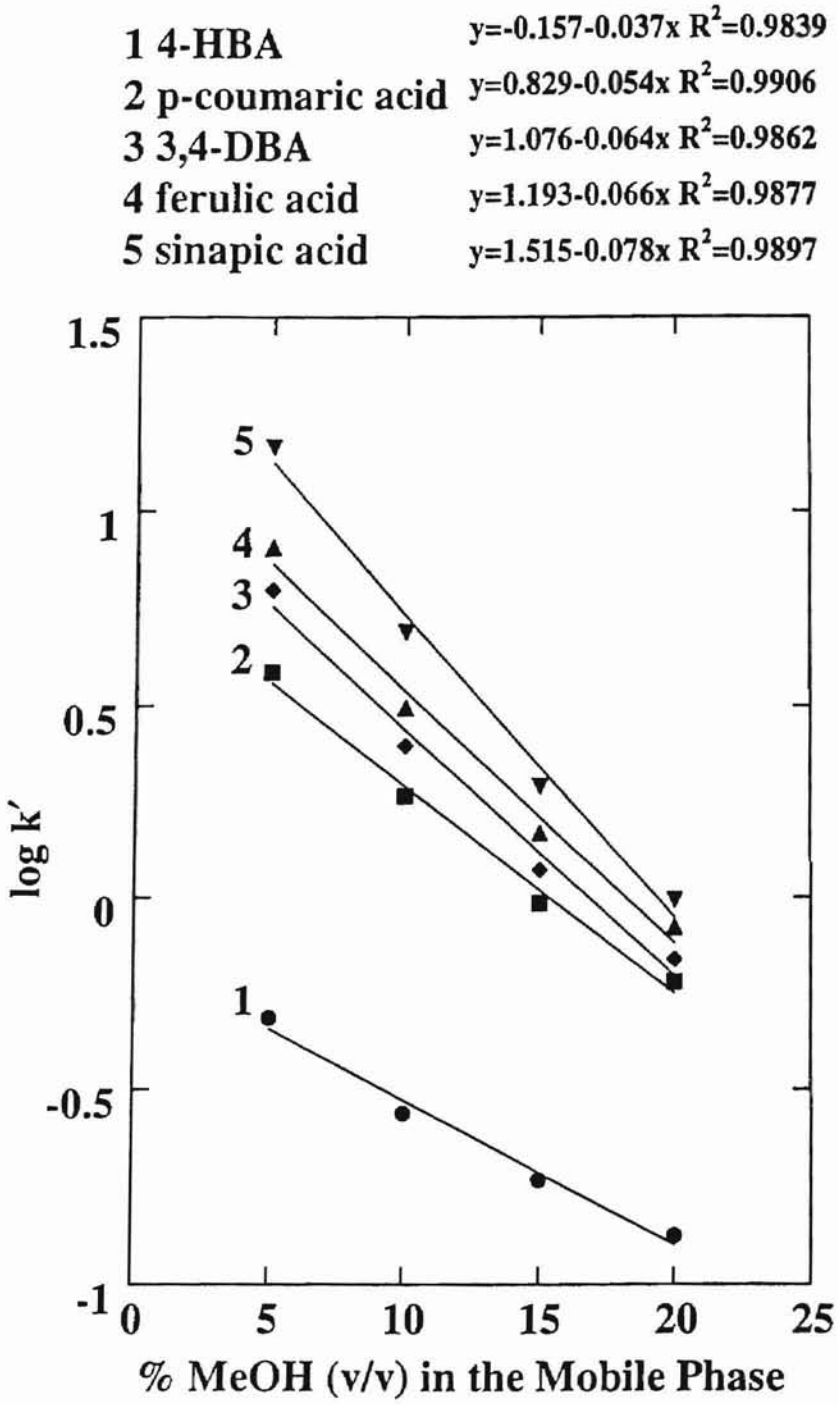


Figure 5. Plots of logarithmic retention factor (log k') versus percent methanol in the mobile phase. Mobile phase, 10 mM $NH_4H_2PO_4$, pH 6.0 at various methanol content. Curves: 1, 4-HBA; 2, *p*-coumaric acid; 3, 3,4-DBA; 4, ferulic acid; 5, sinapic acid.

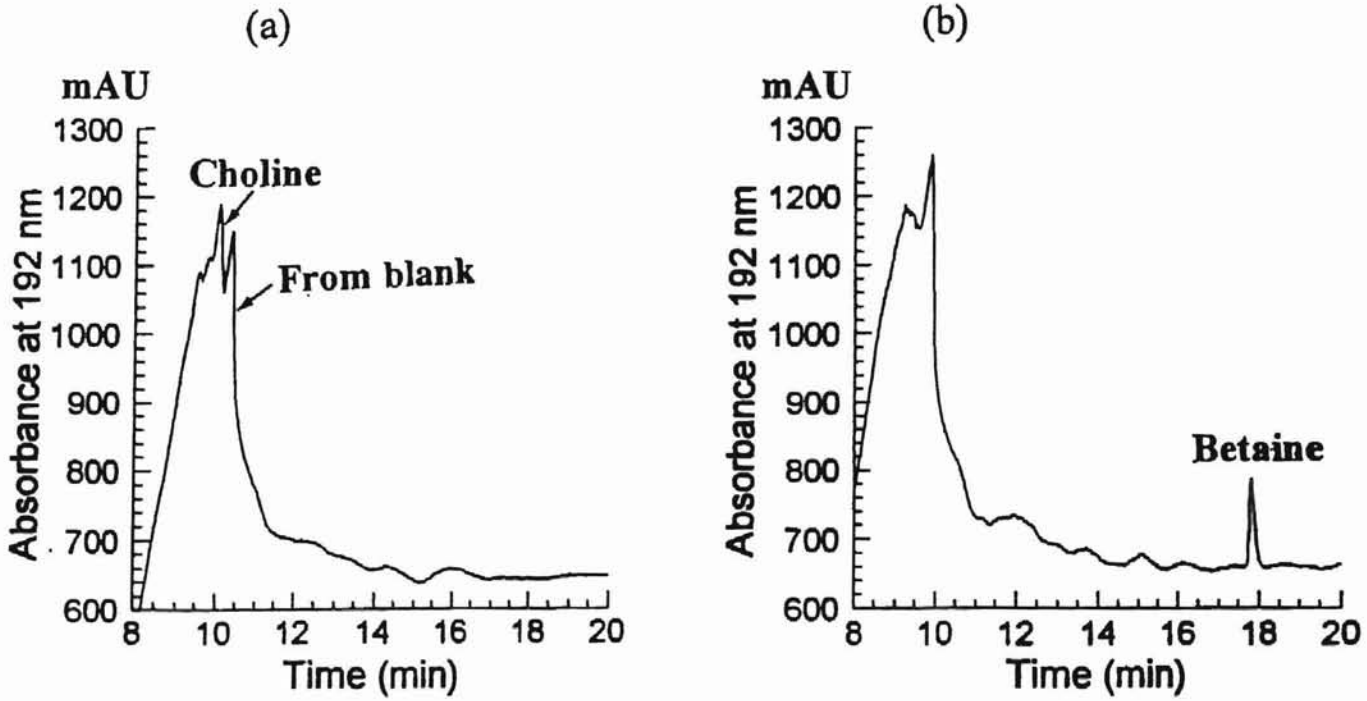


Figure 6. Chromatograms of (a) standard choline and (b) betaine obtained by NPC. Column, 150 x 4.6 mm I.D. packed with 5 μm silica particles; flow rate, 1.0 mL/min; 20 min linear gradient elution from 100% A_2 to 100% B_2.

Figure 7 shows the chromatograms corresponding to the analysis of rapeseed extract before and after 2 hours of base hydrolysis, respectively. As can be seen in this figure, the peak eluting at 2.1 min disappeared while a large new peak eluting at 9.6 min appeared. When spiking the extract with standard sinapic acid, the sinapic acid peak coeluted with that eluting at 9.6 min which increased proportionally in size indicating that the peak generated by base hydrolysis is perhaps sinapic acid. Furthermore, the UV spectra from 190 nm to 400 nm generated by the diode array detector of the peak eluting at 9.6 min obtained upon base hydrolysis and the peak of standard sinapic acid are exactly the same, see Fig. 8. From this experiment it can be assumed that the large peak is sinapic acid and the peak eluting at 2.1 min is sinapine whose spectrum obtained by DAD is shown in Fig. 8. On the other hand, 4-HBA, 3,4-DBA, *p*-coumaric acid, ferulic acid do not seem to exist in considerable amount compared with sinapic acid. Thus, sinapic acid is the dominant phenolic acid existing in rapeseed extract after base hydrolysis.

The base hydrolysis of sinapine is completed in almost 30 min. The time course for the hydrolysis is shown in Fig. 9. The peak area of sinapic acid increased dramatically during the first 30 min and after that it increased slowly. This means hydrolysis of sinapine occurred mostly in the first 30 min. However, 2-hours of hydrolysis time was chosen to make sure all the sinapine was converted to sinapic acid.

As explained in Experimental section, the determination of sinapic acid in the base hydrolyzate of the rapeseed extract was based on the standard calibration curve using ferulic acid as the internal standard. The plot of peak height ratio of sinapic acid to ferulic acid (I.S.) versus the concentration of sinapic acid was a straight line ($y = 0.081x - 0.052$, $R^2 = 0.9977$). From this standard calibration curve, the amount of sinapic acid in the extract was determined to be 7.3 μmol per gram of rapeseed.

Since sinapic acid is the base hydrolysis product of sinapine, the molar concentration of sinapic acid equals to that of sinapine. Thus, in each gram of rapeseed, there is 7.3 μmol of sinapine assuming that hydrolysis goes to completion. This seems to

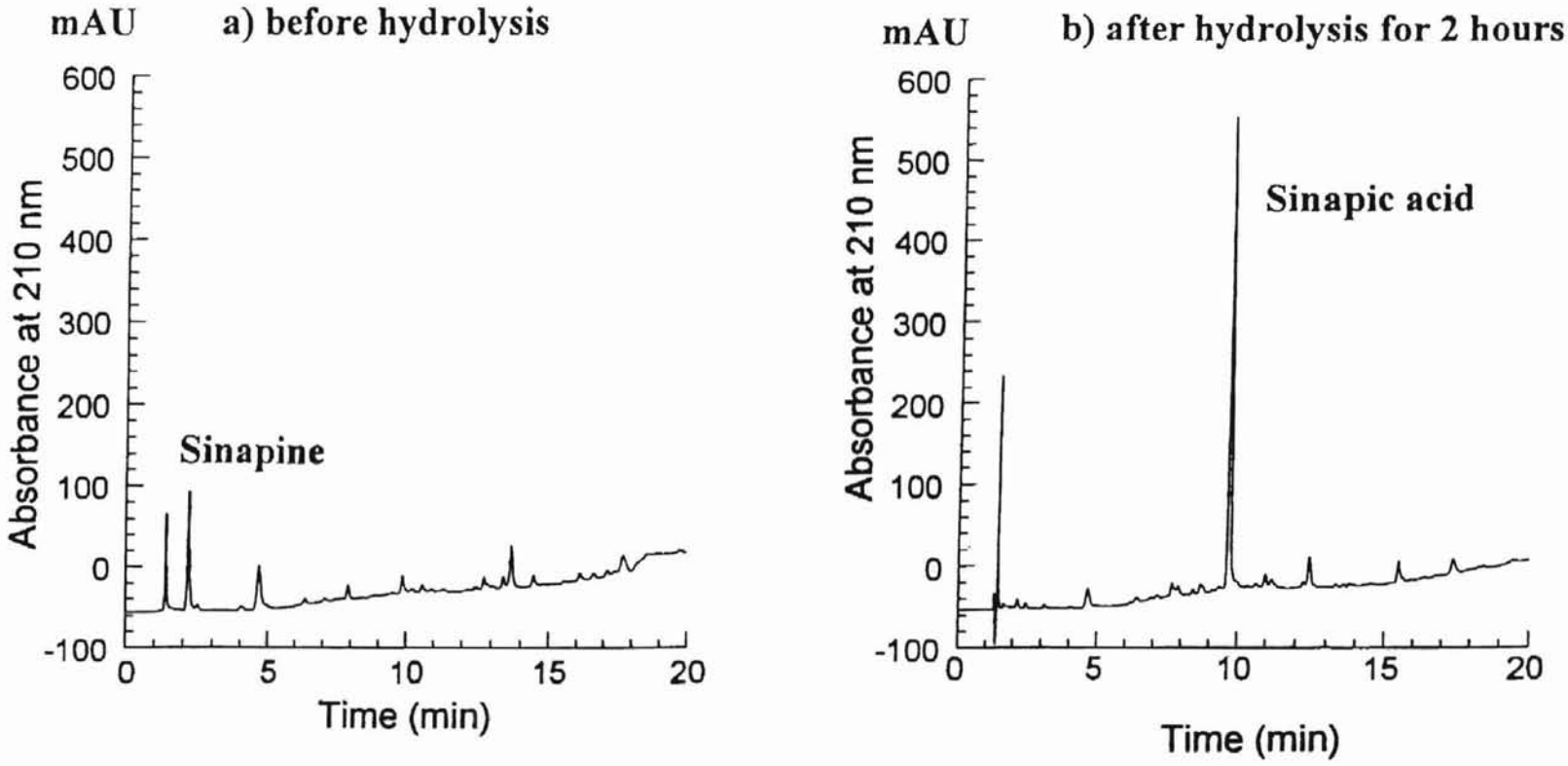


Figure 7. Chromatograms of (a) the intact and (b) the base hydrolyzed for 2 hrs of rapeseed extract obtained by RPC. Column, 150 x 4.6 mm I.D. packed with C18-silica. All other conditions as in Fig. 3.

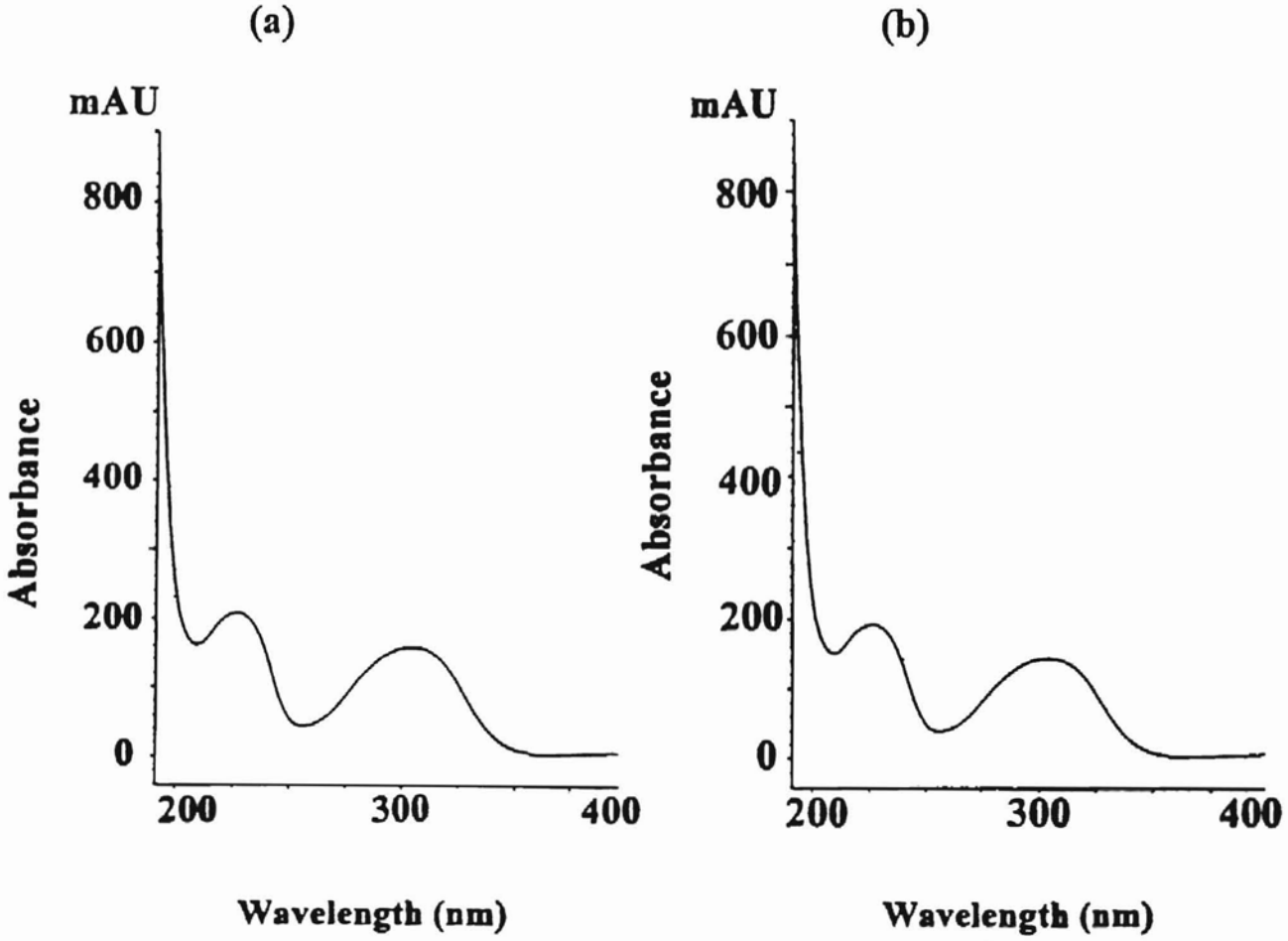
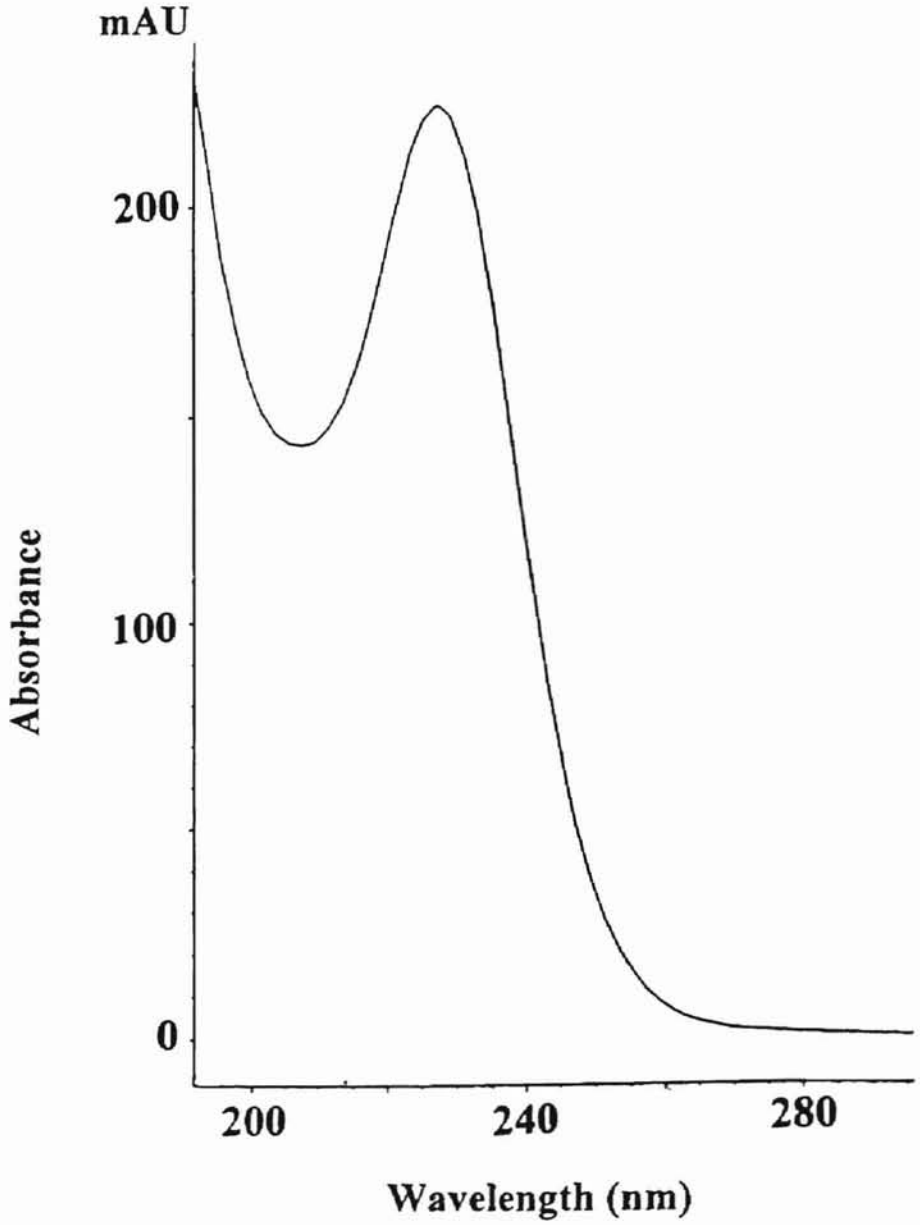


Figure 8. UV-Vis spectra of (a) the standard sinapic acid peak, (b) the peak appearing after base hydrolysis of rapeseed extract as in Fig. 7b and (c) of the peak labeled sinapine in Fig. 7a obtained by the diode array detector used to monitor the column effluent.

(c)



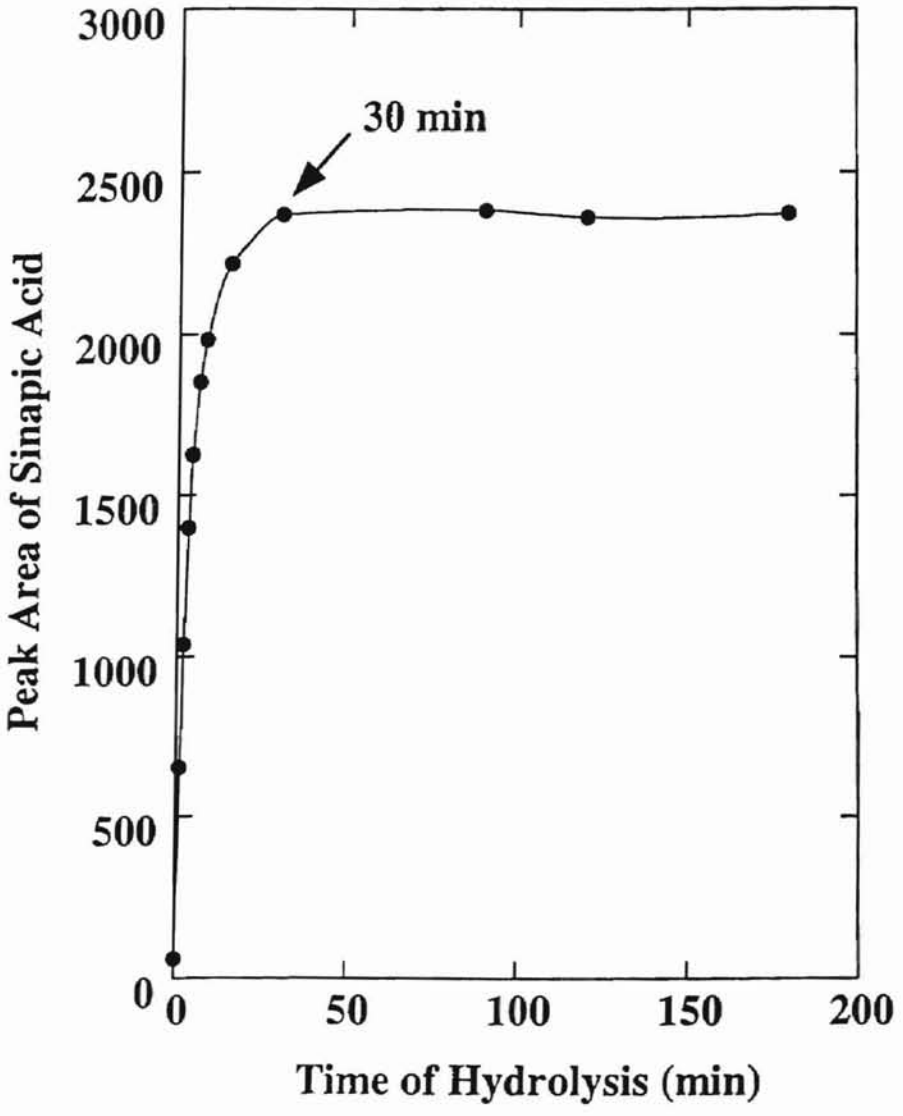


Figure 9. Time course for the appearance of the peak of sinapic acid in the rapeseed extract upon base hydrolysis.

be the case since the peak corresponding to sinapine disappeared totally after 2 hrs of subjecting the rapeseed extract to base hydrolysis.

Enzymatic identification of sinapine through choline oxidase

To further confirm the presence of sinapine in rapeseed, the base hydrolyzed extract was treated with choline oxidase, which converts choline to betaine (see Introduction). In fact, after adding choline oxidase into the base hydrolyzed extract, a new peak appeared at 18 min. The chromatograms obtained in the presence or absence of the choline oxidase are shown in Fig. 10. When spiked with betaine, the extract still yielded one peak at ca. 18 min. That is, the standard betaine peak coeluted with the peak eluting at 18 min. This betaine peak must come from the oxidation of choline by choline oxidase. To further confirm the identity of the peak eluting at 18 min its DAD spectrum was compared with that of standard betaine. Both spectra were identical.

The enzymatic conversion of choline to betaine was found to be slow, and as expected its speed depended on the number of enzyme units added to the reaction. In fact, it took almost a day to completely convert choline to betaine upon adding only 10 units of choline oxidase to a 1.0 mL of the base hydrolyzed rapeseed extract containing ca. 7.0 μmole choline. This time was reduced to almost 2.5 hrs when 50 units of choline oxidase were used.

Thus, sinapine could also be identified through betaine. Alanine was chosen as an internal standard. From the standard calibration curve ($y = 0.1216\ x + 0.0884$, $R^2 = 0.9997$), the amount of betaine generated by the enzymatic treatment of the base hydrolyzed rapeseed was determined to be 7.1 μmol/g of rapeseed. This amount is that

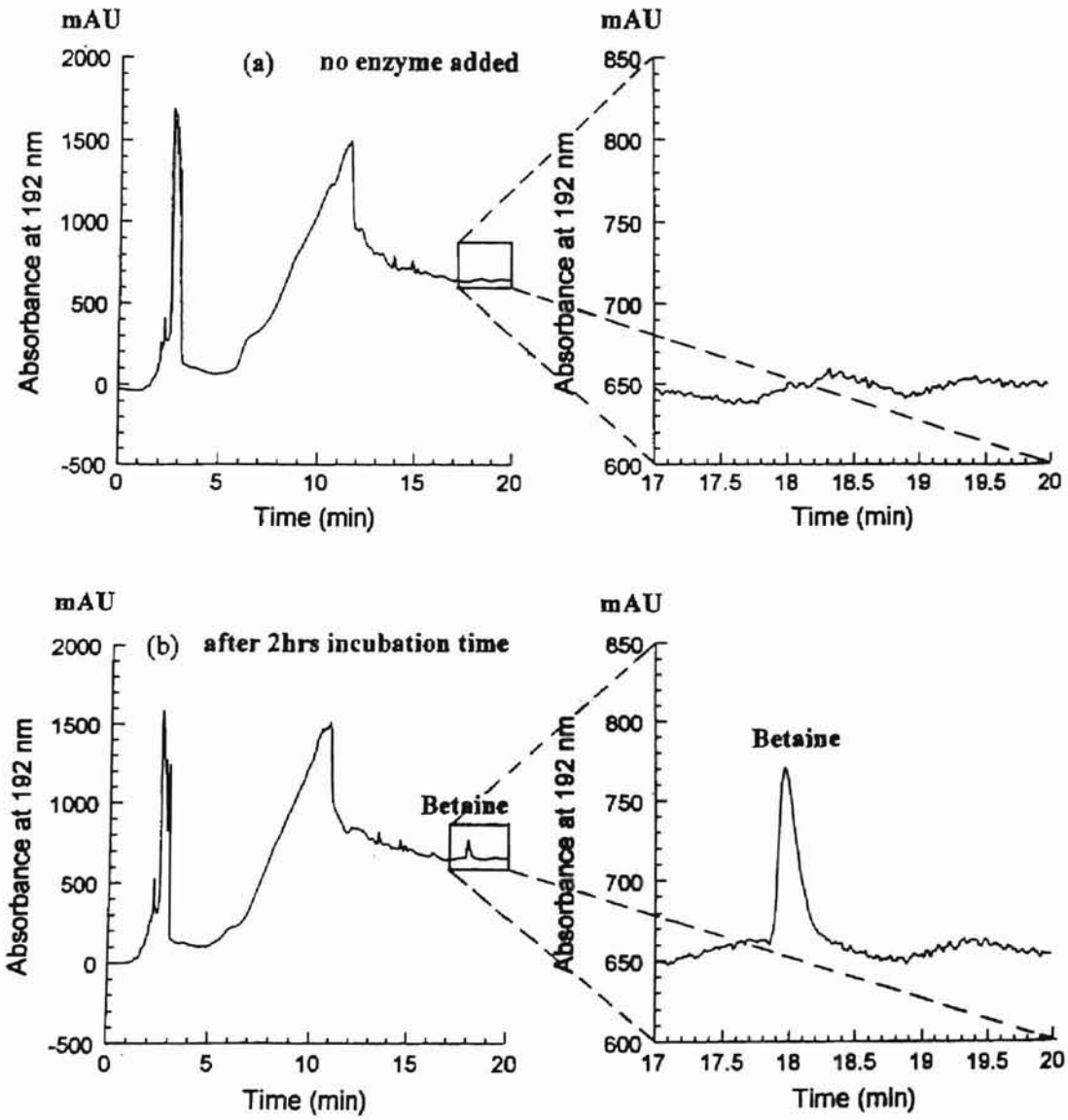


Figure 10. Chromatograms of the base hydrolyzed rapeseed extract in the (a) absence and (b) presence of choline oxidase enzyme. All conditions as in Fig. 7.

of sinapine since 1 mol sinapine produces one mole of choline/betaine. It should be noted that this amount of sinapine falls in the range reported in the literature (15).

Conclusions

Reversed-phase and normal phase chromatography proved useful in the determination of phenolic choline esters via their chemical and enzymatic degradation products (i.e., via phenolic acids and betaine). Sinapine was found to be the major phenolic choline ester constituent of rapeseed. The qualitative and quantitative determination of sinapine in rapeseed can be readily done through the fragments derived from the base hydrolysis and/or the enzymatic treatment of the rapeseed extract. The amount of sinapine was determined to be 7.1-7.3 μmol per gram of rapeseed.

References

1. J.B. Harborne and C.A. Williams, Flavone and flavonol glycosides, in *The Flavonoids,* J.B. Harborne, T.J. Mabry and H. Mabry, Eds. 1975, Academic Press, New York, pp. 376-441.
2. J.B. Harborne and C.A. Williams, Flavone and flavonol glycosides, in *The Flavonoids: Advances in Research,* J.B. Harborne and T.J. Mabry, Eds. 1982, Chapman and Hall, London, pp. 261-311.
3. C.A. Rice-Evans, N.J. Miller and G. Paganga, *Free Rad. Biol. Med.* 1996, *20*, 933-956.
4. K. Herrmann, *Crit. Rev. Food Sci. Nutri.* 1989, *28,* 315-347.
5. M. Friedman, *J. Agric. Food Chem.* 1997, *45,* 1523-1540.
6. G. Block and L. Langseth, *Food Technol.* 1994, July, 80-84.
7. G.A. Block, *Nutr. Rev.* 1992, *50,* 207-213.
8. A.S. Meyer, J.L. Donovan, D.A. Pearson, A.L. Waterhouse and E.N. Frankel, *J. Agric. Food Chem.* 1998, *46,* 1783-1787.
9. J.B. Harborne, General procedures and measurement of total phenolics, in *Plant Phenolics,* J.B. Harborne, Ed. 1989, Academic Press, London, pp. 1-28.
10. M. Naczk, R. Amarowicz, A. Sullivan and F. Shahidi, *Food Chem.* 1998, *62,* 489-502.
11. C. Bjergegaard, L. Ingvardsen and H. Sorensen, *J. Chromatogr. A*, 1993, *653,* 99-108.
12. L. Larsen, T. Nielsen, A. Ploger and H. Sorensen, *J. Chromatogr.*, 1988, *450,* 121-

131.

13. M. Panteghini and R. Bonora, *J. Clin. Chem. Clin. Biochem*. 1984, *22,* 671-676.

14. F.J. Pagani, *Natural Products*, 1982, *45,* 649.

15. J. Bell and A. Shires, *Cana. J. Animal Sci.,* 1982, 62, 557-565.

CHAPTER III

CAPILLARY ELECTROCHROMATOGRAPHY WITH OCTADECYL-SILICA PACKED CAPILLARIES. SEPARATION OF SUBSTITUTED PHENOLS DERIVATIZED WITH A FLUORESCENT CARBAZOLE-9-*N*-ACETIC ACID TAG AND THEIR DETECTION BY UV AND LASER INDUCED FLUORESCENCE

Introduction

Capillary electrochromatography (CEC) employing packed capillary columns with microparticulate stationary phases is increasingly used in various applications (for very recent reviews, see Refs (1, 2)). This is not surprising because CEC combines the features of both liquid chromatography (LC) and capillary electrophoresis (CE), thus offering a unique selectivity complementary to both LC and CE. The mobile phase is transported through the packed capillary by means of electroosmosis instead of pressure. Neutral solutes are separated via differences in partitioning between the mobile and stationary phases and are transported by the electroosmotic flow (EOF). Solutes that partition more in the stationary phase are retarded to a greater extent than those that spent more time partitioning in the mobile phase. On the other hand, charged solutes are

separated by combined effects of partitioning and electrophoresis. The use of EOF as the driving force for differential migration yields a plug flow profile with reduced radial dispersion compared to LC in which a laminar (i.e., parabolic) flow profile is obtained. This leads to a much higher separation efficiency in CEC than in LC, which is about 2 to 3 times greater in the former than in the latter for the same stationary phase particle diameter.

This chapter in concerned with the CEC separation of substituted phenols. To the best of our knowledge, only one research article has dealt so far with the CEC of alkyl substituted phenols (3), and the full potentials of CEC have not been exploited yet in the separation and detection of a wider range of substituted phenols despite the importance of these species. In fact, substituted phenols are of great environmental concern owing to their high toxicity. For instance, chlorinated phenols in water can be the transformation products (i.e., metabolites) of phenoxy alkanoic acid pesticides (4, 5). Also, some of these phenols are released into the environment through many industrial processes. Since phenols are extremely hazardous, a sensitive method for their separation and detection is desirable. UV detection is proved to be not satisfactory. Therefore, precolumn derivatization of the phenols with a fluorescent tag is necessary to bring about this sensitive detection using laser induced fluorescence (LIF). In this way, phenols can be expected to be detected at low concentration levels.

In this study, carbazole-9-N-acetic acid (CRA) was chosen as the fluorescent tag (6, 7) for the substituted phenols under investigation. The retention behaviors of underivatized as well as the CRA derivatized phenols were examined with octadecyl-silica stationary phases and their detection were carried out by both UV and LIF.

Experimental

Reagents and Materials

The structures of the various substituted phenols used in this study are shown in Fig. 1. Phenol was obtained from J.T. Baker Inc. (Phillipsburg, NJ, USA). 2-Chlorophenol, 3-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 2-isopropoxyphenol and 2,2-dimethyl-2,3-dihydrobenzo[b]-7-ol as well as ethyl bromoacetate were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). 1-Naphthol was from Eastman Kodak Co. (Rochester, NY, USA). Carbazole, 4-dimethylaminopyridine (DMAP) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) were from Sigma Chemical Co. (St. Louis, MO, USA). Sodium phosphate monobasic monohydrate was from Mallinckrodt Inc. (Paris, Kentucky, USA). HPLC-grade acetonitrile (ACN) was from Fisher Scientific (Fair Lawn, NJ, USA). HPLC-grade methanol, *N,N*-dimethylformamide (DMF), n-hexane and general reagent acetone were from EM Science (Gibbstown, NJ, USA). General reagent isopropyl alcohol was from Pharmaco (Brookfield, CT, USA). Nucleosil 120-5 silica was obtained from Macherey-Nagel (Düren, Germany). *n*-Octadecyldimethylchlorosilane was from Hüls Petrarch Systems (Bristol, PA, USA). Fused-silica capillaries with an internal diameter of 100 μm were from Polymicro Technologies (Phoenix, AZ, USA).

CEC Instrumentation

Two different instruments were used in this study: An in-house assembled instrument and a Beckman P/ACE instrument (Fullerton, CA, USA). The in-house assembled instrument consisted of a high voltage DC power supply Model CZE1000/PN/R from High Voltage Electronics Corp. (Plainview, NY, USA), a UV-Vis detector Model 200 from Linear Instruments Corp. (Reno, NV, USA) and a CR501

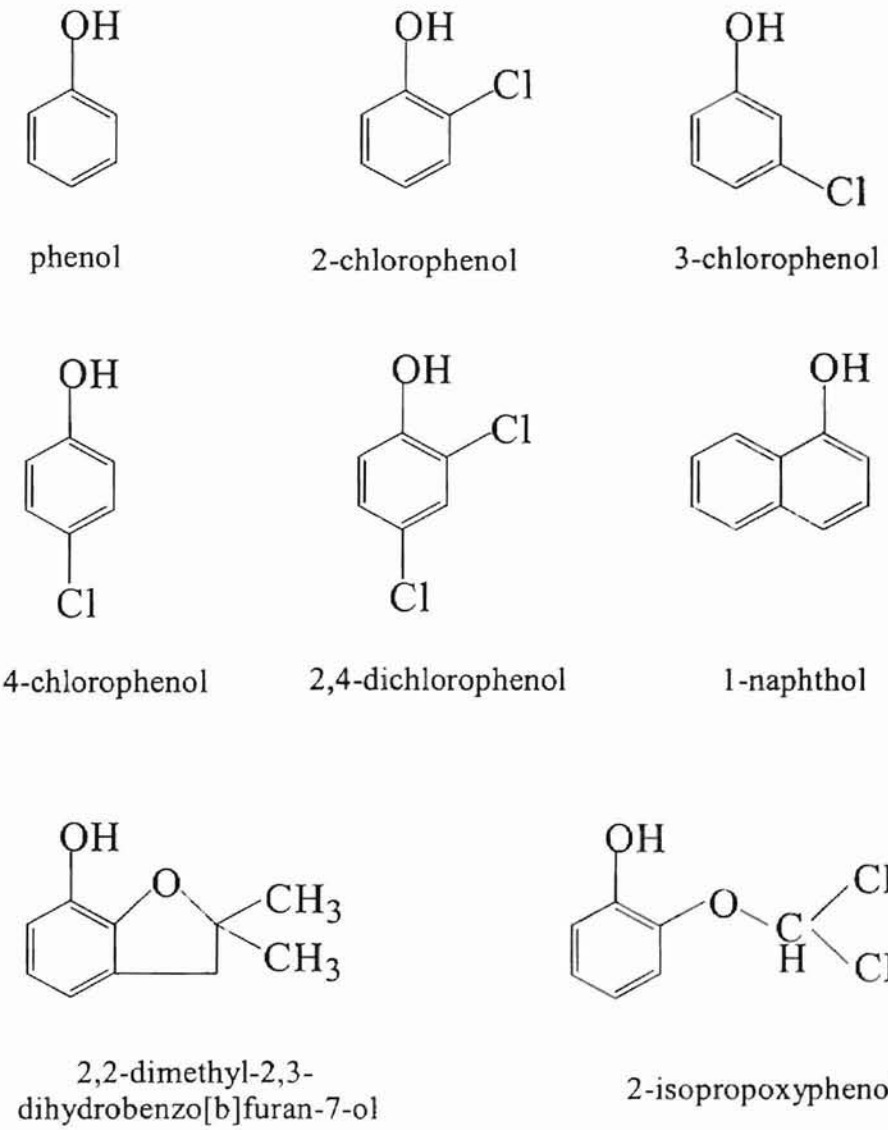


Figure 1. Structures of phenol and some represented substituted phenols.

Chromatopac integrator from Shimadzu Corp. (Kyoto, Japan). The Beckman P/ACE instrument consisted of a Model 5010 equipped with an Omnichrome (Chino, CA, USA) Model 3056-8M He-Cd laser multimode, 8 mW at 325 nm and a data handling system comprising an IBM personal computer and P/ACE station software. An emission band-pass filter of 380 nm ± 2 nm, purchased from Corion (Holliston, MA, USA) was used for the LIF detection of the CRA-derivatized phenols.

Stationary Phase

Nucleosil 120-5 microspherical silica was used as the stationary phase support with a mean particle diameter of 5 μm, a mean pore diameter of 120 Å and a specific surface area of 200 m^2/g. The silica was converted in-house to octadecyl-silica (ODS) according to previous procedures (8-11). Briefly, 2 g of silica and 3 g of octadecyldimethylchloro-silane were added into 30 mL of toluene heated at 109°C. The mixture was kept at the same temperature for 24 hrs in a thermostatic oil-bath and then left to cool at room temperature. The supernatant was drained, and the surface-modified silica was washed with toluene and acetone 3 times each, respectively. The product (ODS) was transferred to a petri dish and left to air dry.

Column Fabrication, Packing and Conditioning

Before packing the capillary column, an outlet frit was made by dipping that end into wet bare Nucleosil 120-5 silica followed by sintering the end in a Bunsen burner for 1–2 min (9, 10). A detection window of 6~8 mm width was made by burning off the polyimide protection layer in the Bunsen burner for 2–3 s followed by wiping the burned polyimide with a soft paper wetted with methanol. The open inlet end was then attached

to a stainless steel slurry reservoir of 3.0 cm × 4.6 mm ID by an Upchurch (Temecula, CA, USA) finger tight capillary fitting. A slurry was made by weighting 0.05 g of ODS into 1 mL of acetone. The capillary and the reservoir were then mounted onto the column packer. The packing pressure was increased gradually to 3000 psi and maintained constant at 3000 psi for 1 hr. The pressure on the packed capillary was then reduced gradually to 0 psi. Thereafter, the column was disconnected and washed with degassed DI water for 1 hr. After this washing step, an empty space was formed at the inlet of the capillary. This empty space was cut out and the inlet end was dipped again in bare Nucleosil 120-5 silica, and a temporary inlet frit was made in the same way as the outlet frit.

Acetonitrile in a microsyringe was manually pushed into the capillary column with the assistance of an in-house built device that resembles a Hoffman tubing clamp. After an overnight washing with ACN, an empty space was formed right after the inlet frit. This temporary inlet frit was then cut out, and the column was again washed with degassed DI water followed by cutting the empty space and making a new permanent inlet frit. ACN was pushed into the column for 1 hr. After this treatment, the column is more durable and can last more than 2 weeks without many air bubble problems.

Before analysis, the column was equilibrated with the mobile phase from 1 kV to 15 kV with a 2 kV increment every 15 min. In this study, two columns with different sizes were made. One was 15 cm from the detection window to the outlet frit with a 40 cm total length (i.e., 25 cm effective length) for the study using the in-house assembled instrument equipped with a UV detector. The other column was 7 cm from the detection window to the outlet frit with a 27 cm total length (i.e., 20 cm effective length) for the study using the Beckman instrument equipped with a LIF detector.

Synthesis of CRA and Derivatization of Phenols

Synthesis of CRA

The CRA was synthesized according to the procedure of Fan et al. (7). Briefly, 4.10 g of carbazole were dissolved in 10.0 mL of DMF followed by adding 5.0 mL of ethyl bromoacetate and 4.0 g of potassium hydroxide. The mixture was heated in an oil-bath for 20 min and extracted with 20.0 mL of 4.0 M potassium hydroxide. 2.0 M hydrochloric acid was then added to adjust the pH of the extracted solution to 2.0. The precipitate was filtered off and washed with 9:1 (v/v) of water/ethanol. The product was extracted from toluene to yield a yellow solid. The synthesis process is shown in Fig. 2a.

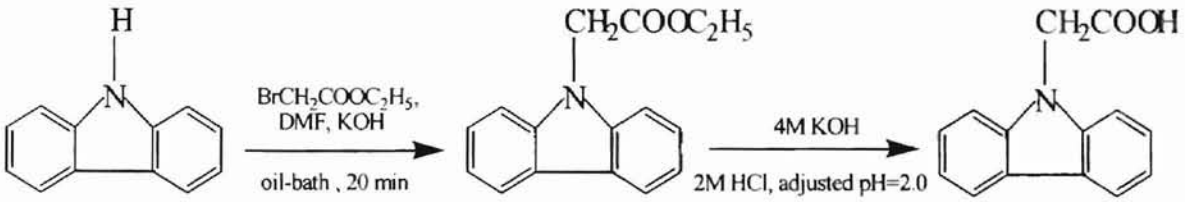
Derivatization of the phenols with CRA

Typically, 200 μL of 10^{-2} M solution of each analyte (i.e., phenol and substituted phenols) were added into a 1mL reaction vial. To each vial, 50 μL of DMAP(50mg/mL), 100 μL of EDAC (25mg/mL) and 100 μL of CRA (25mg/mL) were added successively. The derivatization reaction is shown in Fig. 2b. The mixture was heated at 60°C for 30 min in a Type 17600 Dri-bath (Dubuque, IA, USA). This was followed by the following extraction process: 5.0 mL of *n*-hexane were added into the reaction mixture followed by washing with 4.0 mL each of 1.0 M hydrochloric acid, deionized water, 1.0 M sodium hydroxide and deionized water, respectively (6). The organic phase was separated and evaporated to dryness in a Savant vapor trap (Savant, Holbrook, NY, USA).

Chromatographic Conditions

The mobile phase consisted of various mixtures of ACN/buffer (85/15, 80/20, 75/25, 70/30 v/v). The buffer consisted of 5 mM ammonium phosphate monobasic, pH 6.0. The running voltage was kept at 15 kV unless otherwise indicated. All the samples were electrokinetically injected into the system at 1 kV for 10 s unless otherwise mentioned.

(a)



(b)

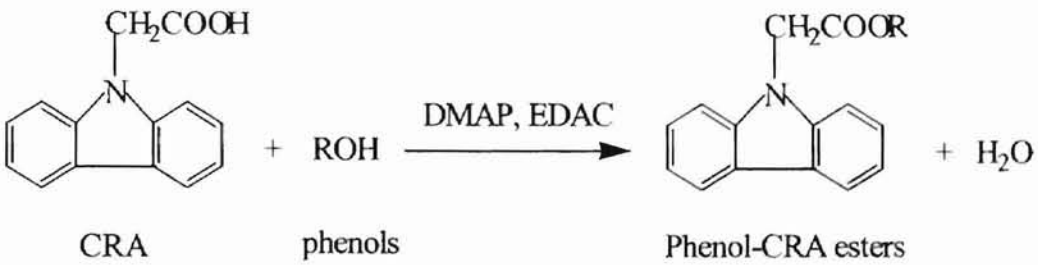


Figure 2. Schemes for (a) the synthesis of CRA and (b) derivatization reaction of phenol and substituted phenols with CRA.

Results and Discussion

Column Evaluation

A mixture of test solutes consisting of the homologous series benzene, toluene, ethylbenzene, propylbenzene and butylbenzene was injected into the system to evaluate the performance of the ODS capillary column. Figure 3 shows a typical electrochromatogram of the test mixture which yielded an average plate count of 26,300, which corresponds to 105,200 plates/m. This high plate count is indicative of a successful silica surface modification as well as a good column fabrication and packing. Under the mobile phase condition used in Fig. 3 and at 15 kV running voltage, an average mobile phase linear velocity (i.e. EOF velocity) of 0.73 mm/s was obtained. This moderate EOF should allow the rapid separation of the phenolic compounds under investigation. The retention factor k' of the test solutes were 0.40, 0.48, 0.57, 0.69 and 0.85 for benzene, toluene, ethylbenzene, propylbenzene and butylbenzene, respectively. This magnitude of retention is indicative of a fairly good surface coverage of octadecyl ligand bonded to the silica surface.

CEC of Underivatized Phenols

A series of CEC runs were performed on the underivatized phenol solutes with mobile phases at varying acetonitrile/buffer (v/v) composition including 85/15, 80/20, 75/25 to 70/30. Figure 4 shows plots of the retention factor, k', of the solutes versus the %ACN in the mobile phase. The column exhibits a reversed-phase behavior as manifested from the decrease in the numerical value of k' with increasing %ACN in the mobile phase. As can be seen in Fig. 4, decreasing the %ACN from 85% to 70% resulted in the partial separation of 2,4-dichlorophenol and 2-isopropoxyphenol while the three monochlorophenol isomers were not separated in the entire %ACN range studied. Figure 5 shows the CEC electrochromatograms of the underivatized phenols. The limits of detection (LOD) were measured from successive dilution of the phenol solutions and approximated when a signal-to-noise ratio of 3-1 was obtained. The LOD of these

phenols are quite high (~10^{-3} M) in the UV at 254 nm (see Table 1). Thus, there is a need for precolumn derivatization to allow their detection at low levels.

CEC of Derivatized Phenols

A series of CEC runs were performed on the CRA derivatized phenols by varying the ACN/buffer (v/v) content of the mobile phase at the following proportions: 85/15, 80/20, 75/25 and 70/30. Figure 6 shows the plots of k' of the CRA derivatized phenols vs. % ACN in the mobile phase. As can be seen in Fig. 6, the three monochlorophenol isomers derivatized with CRA were separated after the %ACN of the mobile phase was decreased to 70%. The isomers were separated in the order of *ortho*, *para* and *meta* forms. It is also found that 2-isopropoxyphenol-CRA and 2,2-dimethyl-2,3-dihydrobenzo[b]-7-ol -CRA derivatives coeluted at 85% ACN composition and they were separated at 70% ACN composition, while 2-isopropoxyphenol-CRA and 1-naphthol-CRA derivatives were separated at 85% ACN composition and they coeluted at 70% ACN composition. Figure 7 shows the CEC electrochromatograms of the phenol and substituted phenols derivatized with CRA.

The percent conversion of a given phenol solute to its CRA ester derivative was determined by CEC analysis (as in the preceding section) of two aliquots of the given phenol at the same solute concentration where one aliquot consisted of the underivatized solute while the other aliquot was derivatized with CRA. The comparison of the peak height of the analyte observed on the electrochromatogram of the underivatized aliquot to the peak height of the analyte obtained on the electrochromatogram of the derivatized aliquot permitted the determination of the % of remaining underivatized analyte and in turn the % conversion. Table 1 lists the % conversion of the phenol and substituted

phenols to their CRA ester derivatives. As can be seen in Table 1, the % conversion was the lowest for 2,4-dichlorophenol and 2,2-dimethyl-2,3-dihydrobenzo[b] -7-ol, standing at 27 and 29%, respectively. The low % conversion with the dichloro substituted phenol can be explained by the inductive electron withdrawing effect of chlorine which is caused by its relatively high electronegativity. This inductive effect renders the electrons on the oxygen in the phenolic group less available for reaction with the carbonyl group of the CRA. The bulkiness of the benzofuranol group is thought to cause the low % conversion for the 2,2-dimethyl-2,3-dihydrobenzo[b]-7-ol to its CRA ester derivative. On the other hand the relatively high percent conversion for 2-isopropoxyphenol (81%) is believed to be the result of the electron donating effect of the isopropoxy group thus making the electrons on the oxygen of the phenolic group more available for reaction with the carbonyl group of the CRA.

The measurement of percent conversion was essential for the determination of the exact limit of detection (LOD) of the CRA-phenol derivatives. As can be seen in Table 1, the CRA derivatization allowed the sensitive UV detection of phenols at 254 nm and yielded LOD's in the 10^{-5} to 10^{-6} M level. The LOD values were measured from successive dilution of the derivatization reaction for each solute. The LODs reported in Table 1 were obtained by multiplying the analyte concentration in the most dilute mixture by the % conversion. The LODs of the CRA derivatized phenols are about 30 ~ 350 times lower than those of underivatized phenols in the UV detection. These LODs could be further decreased by prolonging the time of injection due to the concentrating effect of CEC (see Fig. 8) for relatively retained solutes such as the CRA-phenols as was previously reported by Yang and El Rassi (11, 12) for other neutral compounds. In fact, prolonging the injection time from 10 s to 60 s increased the signal by a factor of at least

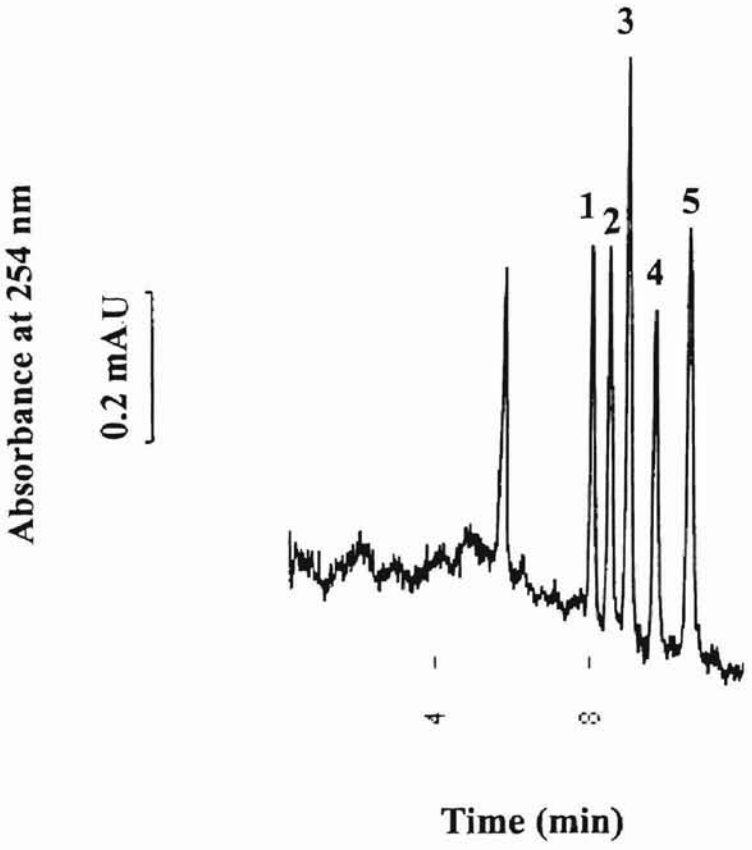


Figure 3. A typical electrochromatogram of benzene and alkylbenzenes using UV detection. Capillary column, 25 cm/40 cm × 100 μm, packed with ODS under 3000 psi for 1 hr; mobile phase, hydroorganic solution made up of 20% (v/v) of 5 mM NaH_2PO_4, pH 6.0, and 80% ACN (v/v); running voltage, 15 kV; injection voltage, 1 kV; injection time, 10 s; detection window at 25 cm from column inlet; detection wavelength, 254 nm. Solutes: 1, benzene; 2, toluene; 3, ethylbenzene; 4, propylbenzene; 5, butylbenzene.

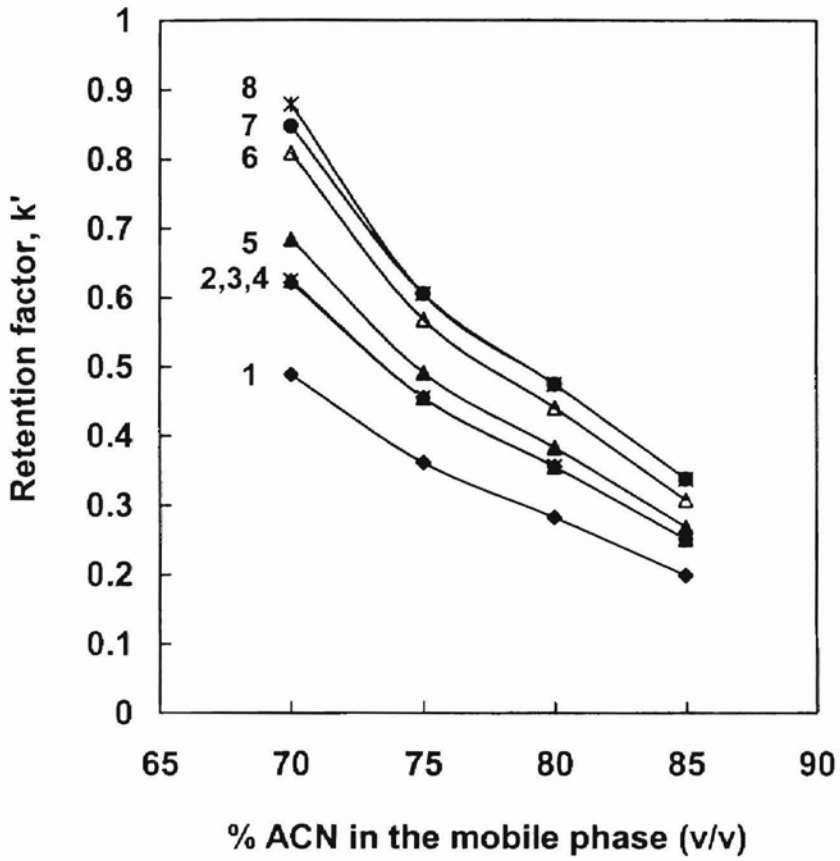


Figure 4. Plots retention factor k' of underivatized phenols versus percent of ACN in the mobile phase (V/V). Mobile phase, 5 mM NaH_2PO_4, pH 6.0 at various ACN content. All other conditions as in Fig. 3. Curves: 1, phenol; 2, 2–chlorophenol; 3, 4-chlorophenol; 4, 3-chlorophenol; 5, 2,2-dimethyl-2,3-dihydrobenzo[b]-7-ol ; 6, 1-naphthol; 7, 2-isopropoxyphenol; 8, 2,4-dichlorophenol.

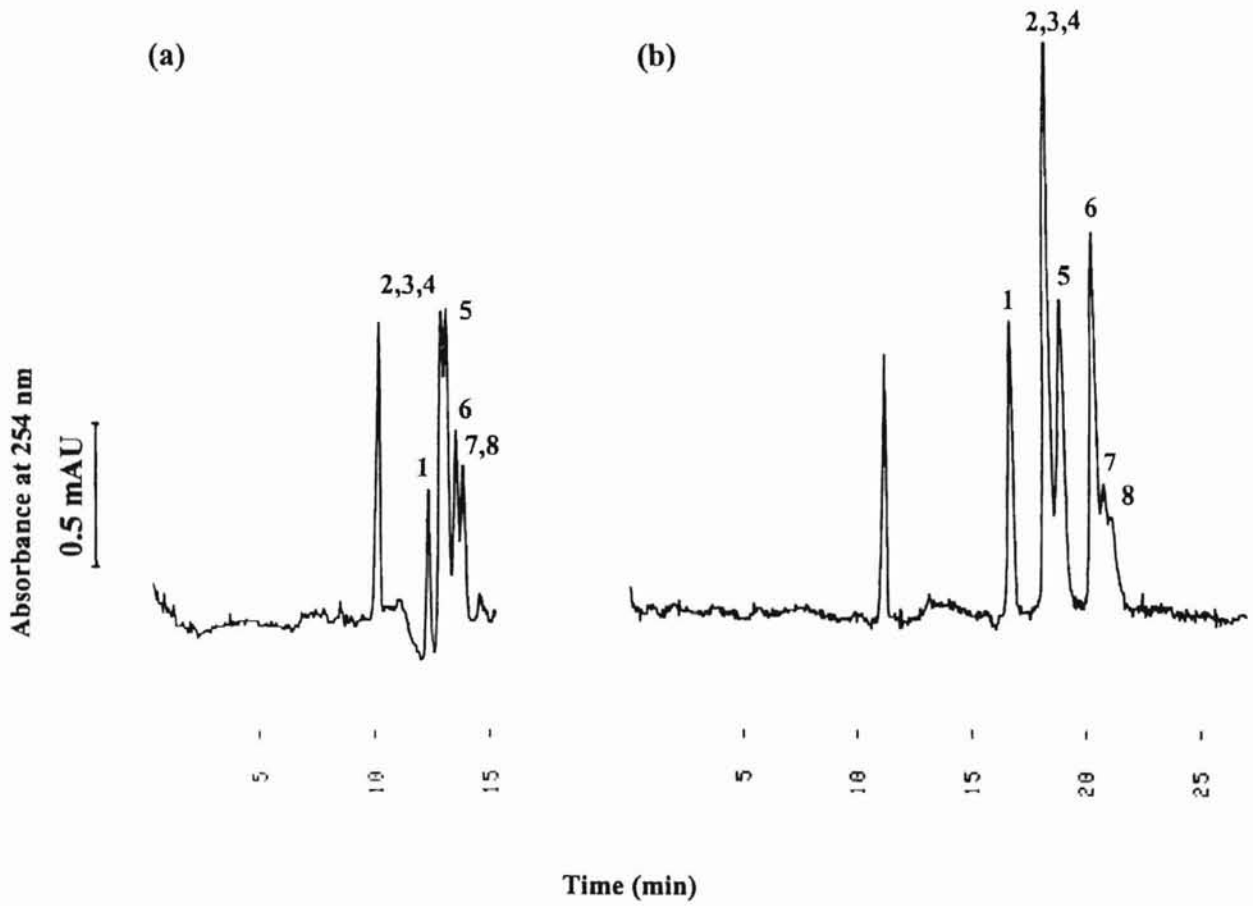


Figure 5. Electrochromatograms of underivatized phenols using UV detection. Mobile phase: (a) 85% (v/v) of ACN and 15% (v/v) 5 mM NaH_2PO_4 (pH 6.0); (b) 70% (v/v) of ACN and 30% (v/v) 5 mM NaH_2PO_4 (pH 6.0); All other conditions as in Fig. 3. Solutes: 1, phenol; 2, 2–chlorophenol; 3, 4-chlorophenol; 4, 3-chlorophenol; 5, 2,2-dimethyl-2,3-dihydrobenzo[b]-7-ol ; 6, 1-naphthol; 7, 2-isopropoxyphenol; 8, 2,4-dichlorophenol.

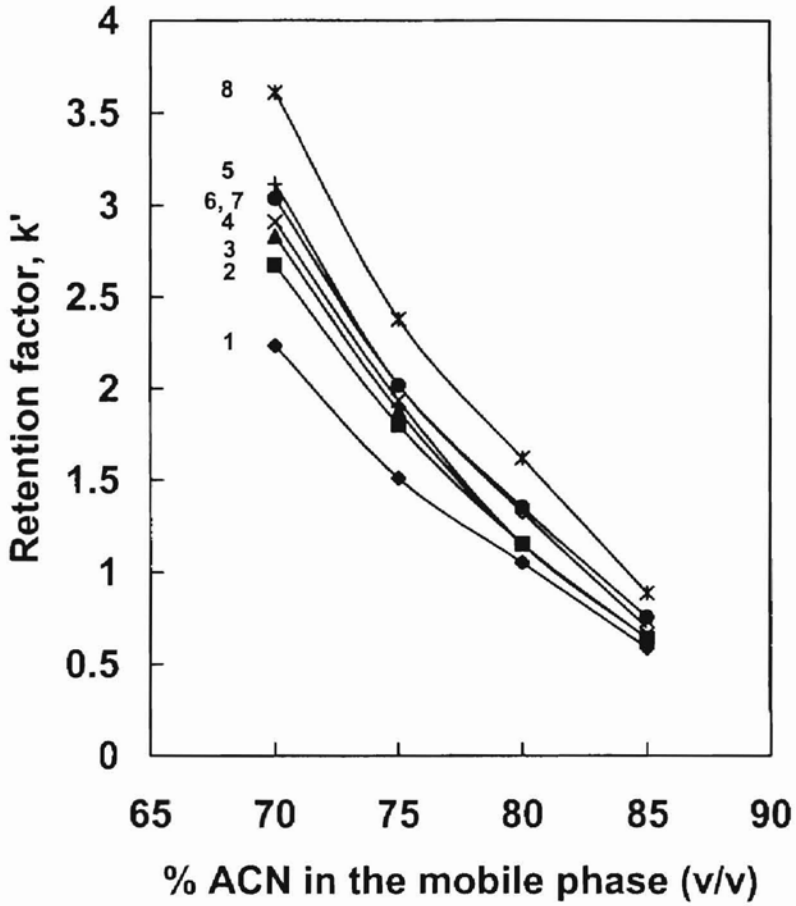


Figure 6. Plots of retention factor k' of derivatized phenols versus percent of ACN in the mobile phase (v/v). All conditions as in Fig. 4. Curves: 1, phenol-CRA; 2, 2-chlorophenol-CRA; 3, 4-chlorophenol-CRA; 4, 3-chlorophenol-CRA; 5, 2,2-dimethyl-2,3-dihydrobenzo[b]-7-ol -CRA; 6, 1-naphthol-CRA; 7, 2-isopropoxyphenol-CRA; 8, 2,4-dichlorophenol-CRA.

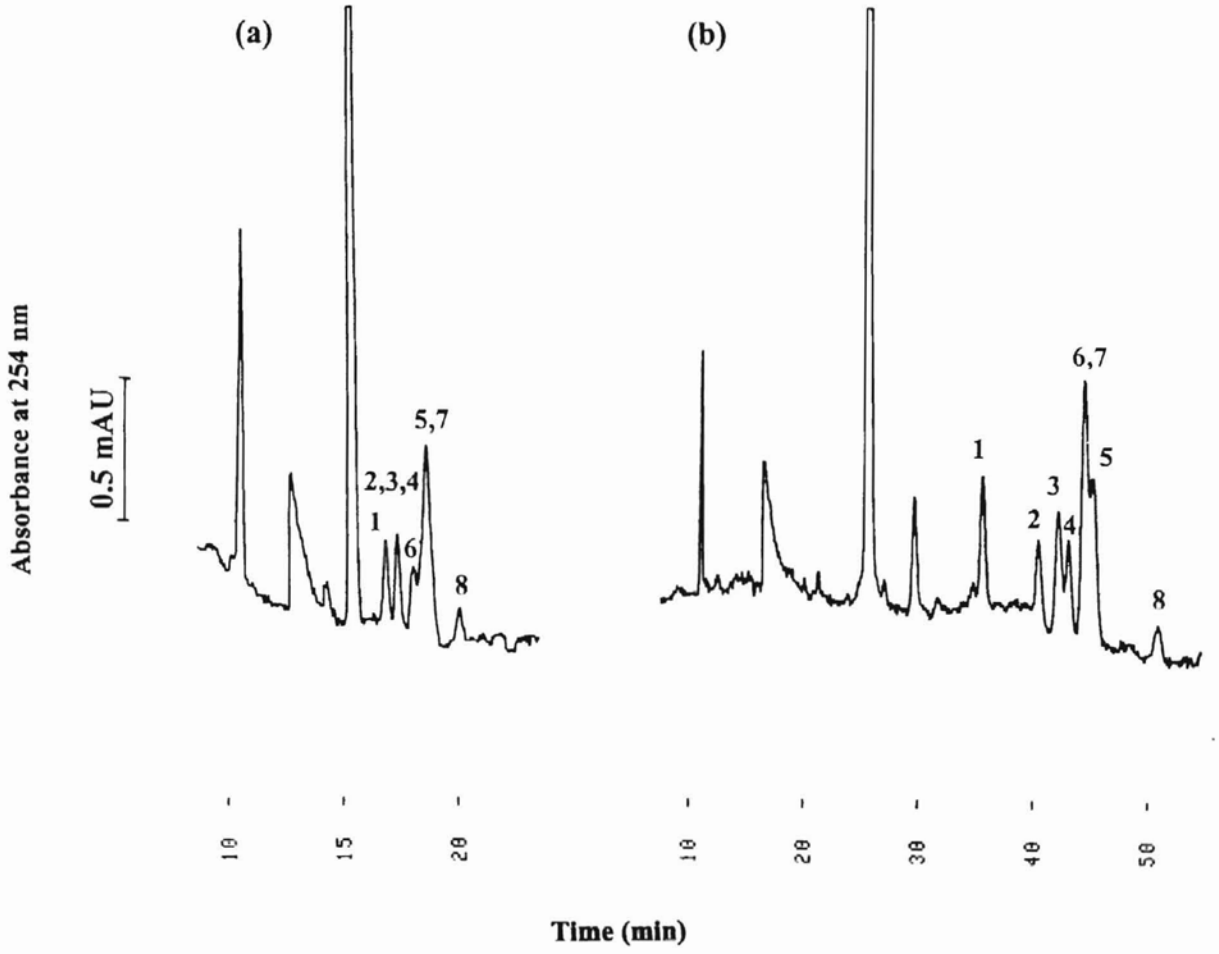


Figure 7. Electrochromatograms of derivatized phenols using UV detection. Mobile phase, (a) 85% (v/v) of ACN and 15% (v/v) 5 mM NaH_2PO_4 (pH 6.0); (b) 70% (v/v) of ACN and 30% (v/v) 5 mM NaH_2PO_4 (pH 6.0). All other conditions as in Fig. 3. Solutes: 1, phenol-CRA; 2, 2-chlorophenol-CRA; 3. 4-chlorophenol-CRA; 4, 3-chlorophenol-CRA; 5, 2,2-dimethyl-2,3-dihydrobenzo[b]-7-ol -CRA; 6, 1-naphthol-CRA; 7, 2-isopropoxyphenol-CRA; 8, 2,4-dichlorophenol-CRA.

Table 1. Percent conversion of phenols to their CRA derivatives and LOD of underivatized phenols in UV @ 254nm and LOD of CRA derivatized phenols in UV @ 254nm and in LIF using He/Cd laser 325 nm as the excitation source.

Solute	LOD (M) Underivatized UV @ 254 nm	% Conversion	LOD (M) CRA-derivatives UV @ 254 nm	LOD (M) CRA-derivatives LIF
phenol	1.2×10^{-3}	48%	1.4×10^{-5}	1.4×10^{-6}
2-chlorophenol	4.4×10^{-4}	46%	2.9×10^{-6}	5.8×10^{-7}
2,2-dimethyl-2,3-dihydrobenzo [b]furan-7-ol	2.2×10^{-4}	29%	4.8×10^{-6}	6.8×10^{-7}
1- naphthol	3.8×10^{-4}	51%	1.3×10^{-5}	1.8×10^{-7}
2-isopropoxyphenol	5.3×10^{-3}	81%	1.5×10^{-5}	3.0×10^{-7}
2,4-dichlorophenol	2.0×10^{-3}	27%	1.6×10^{-5}	1.2×10^{-5}

3. However, there is a limit for prolonging the injection time, since at 60 sec the resolution between 3-chloro- and 4-chlorophenol CRA esters decreased due to an increase in band broadening, see Fig. 8.

Figure 9 shows a typical electrochromatogram of the phenols derivatized with CRA using LIF detection and the same elution conditions as in Fig. 7b, except the

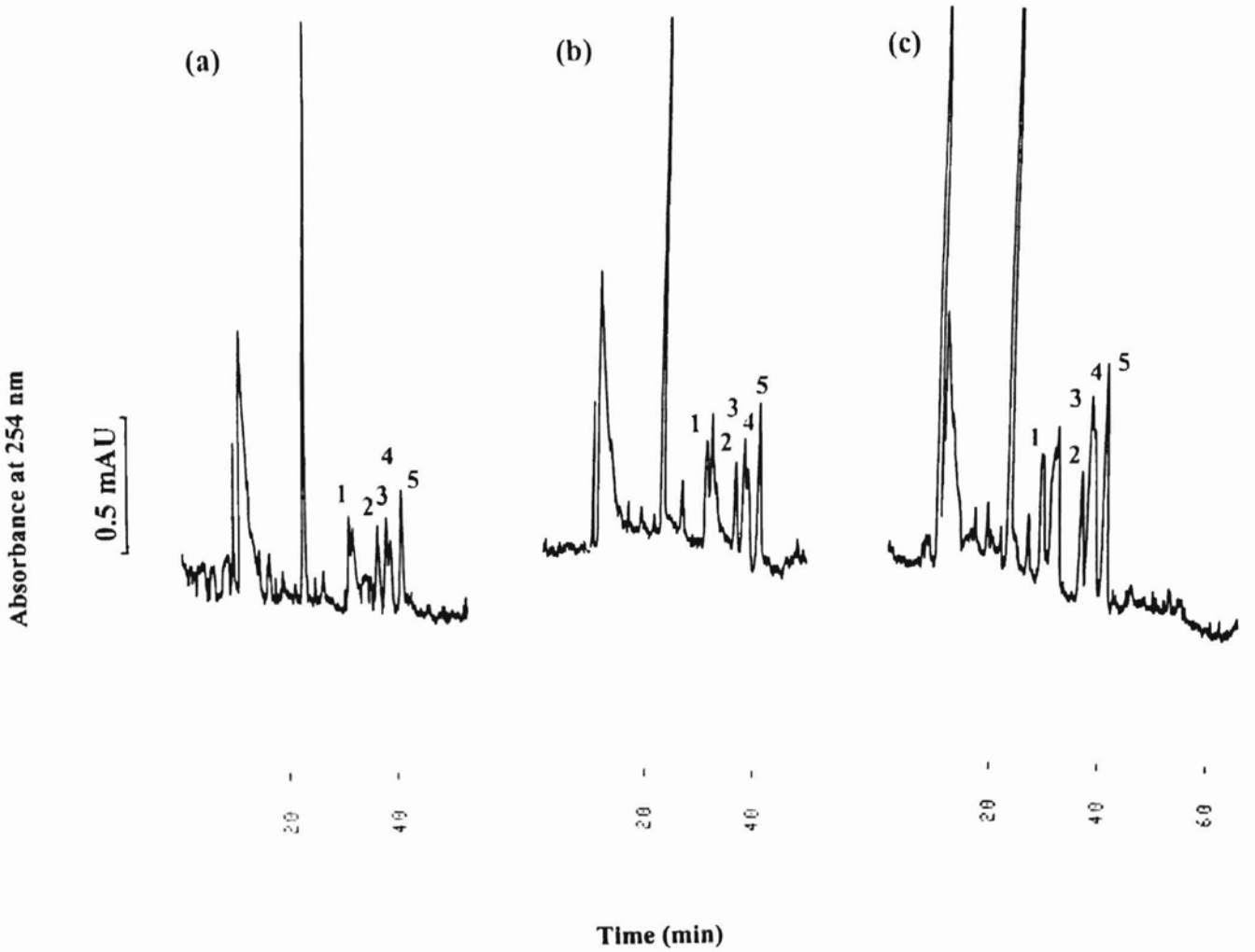


Figure 8. Electrochromatograms of derivatized phenols using UV detection with different injection time. (a) 20 s; (b) 30 s and (c) 60 s. Mobile phase, 70% (v/v) of ACN and 30% (v/v) 5 mM NaH_2PO_4 (pH 6.0). All other conditions as in Fig. 3.

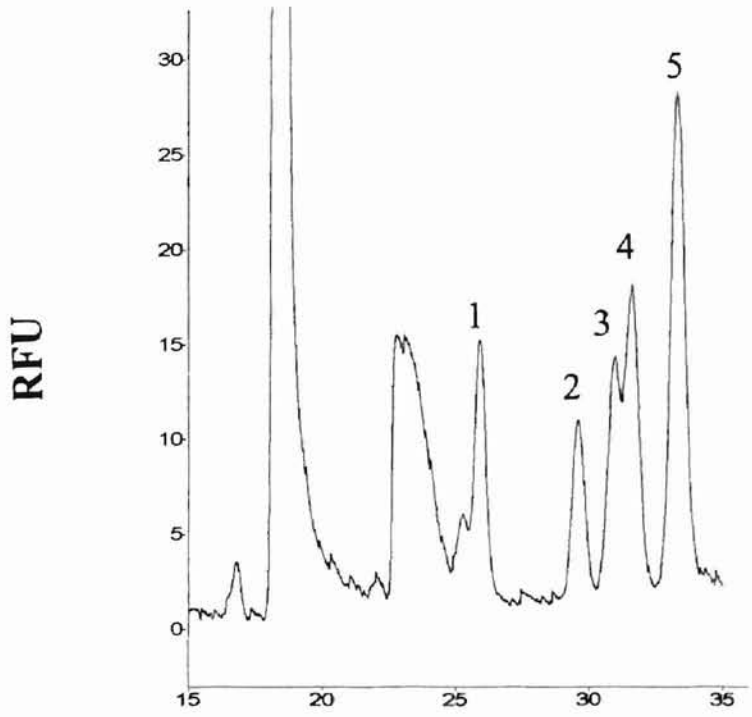


Figure 9. Electrochromatogram of derivatized phenols using LIF detection. Capillary column, 20 cm/27 cm × 100 μm, packed with ODS under 3000 psi for 1 hr; Mobile phase: 70% (v/v) of ACN and 30% (v/v) 5 mM NaH_2PO_4 (pH 6.0); voltage, 10.5 kV; detection window at 20 cm from column inlet; fluorescent detection , excitation at 325 nm, emission at 360 nm. Solutes: 1, phenol-CRA; 2, 2-chlorophenol-CRA; 3, 4-chlorophenol-CRA; 4, 3-chlorophenol-CRA; 5, 2,2-dimethyl-2,3-dihydrobenzo[b]-7-ol – CRA.

capillary column is shorter with an effective length of 20 cm and total length of 27 cm. Despite this fact, the column still exhibited sufficient separation efficiency to allow the resolution of the monochlorophenol isomers.

Table 1 shows the LODs of phenol and substituted phenols derivatized with CRA using LIF detection. The LODs are 1.3 ~ 72 times lower than those obtained using UV detection for the phenol-CRA esters. Phenol-CRA is 10 times more sensitive using LIF than using UV detection. 2-Chlorophenol-CRA ester is 5 times more sensitive in LIF than in UV whereas 2,4-dichlorophenol-CRA ester is only 1.5 times more sensitive in the former than in the latter. This suggests that fluorescence decreases with increasing number of chlorine in the phenol molecule, which is a well known fact (13).

Conclusions

This investigation has shown that CEC is a suitable method for the separation and detection of fluorescently labeled substituted phenols. Not only the derivatization increased the sensitivity of the method by allowing the use of LIF detection but also improved the separation of substituted phenols isomers, e.g., the monochlorophenols. The LODs for the phenol-CRA esters were found to be 30-350 times lower compared to the LODs of underivatized phenols in the UV at 254 nm. In LIF detection, the LODs of the CRA derivatized phenols were found to be 1.3-72 times lower compared to UV at 254 nm. Overall, the LODs were 166-2111 times lower in LIF for the CRA derivatized phenols with respect to the LODs of underivatized phenols in the UV at 254 nm.

References

1. Z. Deyl and I. Miksik, in *Capillary Electrochromatography*, Z. Deyl and F. Svec, Eds. Elsevier Science, Amsterdam, 2001, Chapter 10.
2. A. Dermaux and P. Sandra, *Electrophoresis,* 1999, *20*, 3027-3065.
3. Y.S. Fung and Y.H. Long, *J. Chromatogr. A* 2001, *907*, 301-311.
4. M.F. Gonnord and J. Collet, *J. Chromatogr.* 1993, *645*, 327-336.
5. M. Veningerová, V. Prachar, J. Uhnák, M. Lukácsová and T. Trnovec, *J. Chromatogr. A,* 1994, *657*, 103-110.
6. J. You, B. Zhang, W. Zhang, P. Yang and Y. Zhang, Y., *J. Chromatogr. A* 2001, *909*, 171-182.
7. X. Fan, J. You, J. Kang, Q. Ou and Q. Zhu, *Anal. Chim. Acta* 1998, *367*, 81-91.
8. T. Tegeler and Z. El Rassi, *Anal. Chem.* 2001, *73*, 3365-3372.
9. C. Yang and Z. El Rassi, *Electrophoresis* 1998, *19*, 2061-2067.
10. C. Yang and Z. El Rassi, *Electrophoresis* 1999, *20*, 18-23.
11. C. Yang and Z. El Rassi, *Electrophoresis* 1999, *20*, 2337-2342.
12. C. Yang and Z. El Rassi, *Electrophoresis* 2000, *21*, 1977-1984.
13. D.A. Skoog, F.J. Holler and T.A. Nieman, *Principles of Instrumental Analysis*, Saunders College Publishing, Philadelphia, 1998, pp. 362.

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Master of Science

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